



Antimicrobial Roles for iNKT Cells and GM-CSF in Mycobacterium Tuberculosis Infection

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Antimicrobial Roles for iNKT Cells and GM-CSF
in *Mycobacterium Tuberculosis* Infection

A dissertation presented

by

Alissa Chen Rothchild

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Immunology

Harvard University

Cambridge, Massachusetts

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Antimicrobial Roles for iNKT Cells and GM-CSF in *Mycobacterium Tuberculosis* Infection

ABSTRACT

Despite effective antibiotics, *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis, still infects nearly one-third of the world's population. While key immune factors including CD4⁺ T cells and IFN γ production have been identified, there are still many antimicrobial mechanisms yet to be explored. Here we characterized the role of invariant natural killer T (iNKT) cells and GM-CSF during Mtb infection.

iNKT cells are activated during many infections, but their effector functions are unknown in most cases. We investigated how iNKT cells suppress intracellular Mtb replication. When co-cultured with Mtb-infected macrophages, iNKT cell activation, as measured by CD25 upregulation and IFN γ production, was primarily driven by IL-12 and IL-18. In contrast, iNKT cell control of Mtb growth was CD1d-dependent, and did not require IL-12, IL-18, or IFN γ . In this model, conventional activation markers did not correlate with iNKT cell antimicrobial function during Mtb infection. iNKT cell control of Mtb replication was also independent of TNF and cytolytic activity. Dissociating cytokine-driven activation and CD1d-restricted effector function, we uncovered a novel mediator of iNKT cell antimicrobial activity: GM-CSF. iNKT cells produced GM-CSF in a CD1d-dependent manner during Mtb infection, and GM-CSF was both necessary and sufficient to control Mtb growth.

We next investigated GM-CSF production by other T cell subsets during Mtb infection. GM-CSF production increased in the lung over the course of infection and both innate and adaptive T cells produced GM-CSF. Preliminary results from adoptive transfer experiments using WT and GM-CSF^{-/-} T cells revealed that T cell-derived GM-CSF contributed to control of bacterial

growth in vivo. In our last set of studies, we addressed the mechanism by which GM-CSF leads to antimicrobial activity in macrophages. We found that GM-CSF altered host lipid metabolism within macrophages and this likely contributed to control of bacterial growth in a PPAR γ -dependent manner.

Together, these studies identified GM-CSF as a novel antimicrobial effector function for both iNKT cells and other T cell subsets during Mtb infection and described how GM-CSF altered the intracellular niche for Mtb, preventing bacterial growth. These studies highlight the role for non-classical effector functions during Mtb infection.

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LIST OF ABBREVIATIONS

+	having the indicated characteristic (read as “positive”)
-/-	genetically deficient (read as “knock-out”)
3-NP	3-nitropropionate
α GalCer	α -Galactosylceramide
ANOVA	analysis of variance
APC	antigen presenting cell
β 2m	β 2-microglobulin
BCG	bacillus Calmette-Guérin
BMDM	bone marrow derived macrophage
BMDC	bone marrow derived dendritic cell
BODIPY	4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene
CD	cluster of differentiation
CFU	colony forming units
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DHR 123	dihydrorhodamine 123
dNKT	diverse natural killer T cell
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescent activated cell sorting (flow cytometry)
GM-CSF	granulocyte macrophage colony-stimulating factor
hMDM	human monocyte-derived macrophages
HMNC	hepatic mononuclear cell
IFN	interferon
IL	interleukin
iNKT	invariant natural killer T cell
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
LXR	liver X receptor
MDR-TB	multi-drug resistant Mtb
MHC	major histocompatibility complex
MFI	mean fluorescence intensity
MOI	multiplicity of infection
Mtb	<i>Mycobacterium tuberculosis</i>
M ϕ	macrophage
NO	nitric oxide
oxLDL	oxidized low-density lipoprotein
Pfn	perforin
PPAR	peroxisome proliferator-activated receptor
RNI	reactive nitrogen intermediates
ROS	reactive oxygen species
SEM	standard error of the mean
TB	tuberculosis
TCR	T cell receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
WHO	world health organization
WT	wild-type
XDR-TB	extensively-drug resistant tuberculosis

Introductory Note

This thesis is focused on novel antimicrobial mechanisms that control *Mycobacterium tuberculosis* (Mtb) infection. While this research was initially directed towards understanding the role of iNKT cells during Mtb, subsequent results led to further investigation into a role for GM-CSF.

Chapter 1 includes an overview of both tuberculosis and iNKT cell biology. The introduction begins with a short summary of the global health perspective on tuberculosis and a brief synopsis of the history of tuberculosis disease. Next, there is an overview of what is currently known about the immune response to Mtb. Following this is an introduction into the biology of iNKT cells. To link these topics together, the chapter ends with a discussion on the role for iNKT cells during microbial infection.

Studies on the activation and effector function of iNKT cells during Mtb infection are discussed in **Chapter 2**. This work led to the identification of GM-CSF as a novel iNKT cell antimicrobial effector function and was recently published in the journal *PLoS Pathogens*. After GM-CSF was identified as an iNKT cell effector function during Mtb, follow-up studies were performed to characterize the role of T cell-derived GM-CSF during infection and these are included in **Chapter 3**. This work includes studies of different T cell subsets that express GM-CSF in the lung during infection and analysis of its contribution to bacterial control. **Chapter 4** links GM-CSF as a T cell effector function to a direct cellular mechanism of bacterial control through exploration of the effect of GM-CSF on host lipid metabolism.

Chapter 5 contains a general discussion of results focusing on two main themes. First, iNKT cells offer a tremendous opportunity to study novel effector mechanisms during infection due to the multitude of functions they express and the distinct activation pathway for a given pathogen may be key to understanding this. Second, GM-CSF and its perturbation of host cell metabolism may provide insight into Mtb biology and generate new therapeutic targets that alter metabolic pathways at the host-pathogen interface.

Chapter 1: Introduction

Tuberculosis: a global health problem

History of tuberculosis

*Immunity to *Mycobacterium tuberculosis***

Biology of invariant natural killer T (iNKT) cells

Role for iNKT cells during microbial infection

* Some of the text in this section has been adapted from the author's own writing published in:

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Tuberculosis: a global health problem

Tuberculosis (TB) is one of humankind's oldest infectious diseases¹. Despite evolving alongside us for thousands of years, it continues to be an urgent global public health problem. Nearly one-third of the world's population is infected each year, yet most of these infections remain in a latent state. Once latently infected, individuals carry about a 10% lifetime risk of reactivation of their infection, which accounts for the 8 million people per year who become actively sick with *Mycobacterium tuberculosis* (Mtb), the causative agent of TB, and over 1 million people who die from the disease each year². Mtb causes the second greatest number of deaths worldwide of any infectious agent². Only HIV/AIDS causes more deaths. In the United States, almost 10,000 cases and over 500 deaths are reported each year. 63% of these cases are from foreign-born persons. These rates continue to decline in the U.S. from a peak in 1992³. Importantly, Mtb primarily causes disease and death in young adults, which leads to a significant impact on economic activity in addition to overall morbidity and mortality.

One of the biggest driver's of the tuberculosis epidemic in the modern era has been the emergence of HIV/AIDS. As HIV has spread across many populations, it has fueled the re-emergence of tuberculosis. Tuberculosis is the leading cause of death of people infected with HIV. Co-infected individuals are 30 times more likely to develop active TB than those with single infections². Each year there are over 1 million new cases of TB from HIV-positive individuals and 75% of these cases are in Africa². The World Health Organization (WHO) has set special policy guidelines to try to combat the synergism of these diseases. Intensified case finding for TB cases, preventative isoniazid treatment, and infection control aim to ease the burden of TB disease on HIV-positive individuals⁴.

In addition to HIV-TB co-infection, the other major obstacle to combating tuberculosis is the rise of multi-drug resistant tuberculosis (MDR-TB), strains of bacteria resistant to the two most common first-line therapies, isoniazid and rifampin. With current drug regimens, treatment of tuberculosis requires 6-9 months of combination antibiotic drug therapy. These drugs can cause many painful side effects, leading to failures in treatment adherence and selection for mutants

resistant to antibiotics³. There are currently 450,000 new cases of MDR-TB each year as well as many case reports of extensively drug-resistant tuberculosis (XDR-TB), strains with drug resistance to additional second-line antibiotics². The rise of MDR-TB and XDR-TB has put pressure on the public health community to push forward research of new antibiotic candidates. There is additional anxiety derived from the fact that the antibiotics used to treat tuberculosis were almost entirely developed in the 1950s and 1960s⁵. The recent breakthrough in the development of Bedaquiline in 2012 has added one more drug to the arsenal⁵.

The World Health Organization (WHO) has made fighting the global TB epidemic a priority. The Stop TB WHO Strategy along with DOTS (directed observed treatment, short-course) have saved 22 million lives and decreased the death rate by 45% from 1990 to 2012². Moving forward, the WHO estimates that the global cost of combating TB disease over the ten-year period from 2006-2015 will be \$56 billion. While half of the funding is available, they estimate a \$31 billion funding shortage⁶. WHO guidelines focus on both directly treating and preventing disease as well as improving basic health infrastructure, empowering communities, and investing in resources for health care providers. While these steps will go far towards addressing the basic needs of infected individuals and stemming the wave of transmission, there are still many basic scientific questions remaining that must be addressed by basic research to move forward in therapeutic discovery.

History of tuberculosis

DNA from *Mycobacterium tuberculosis* has been found in human remains that date as far back as 9000 years, in neolithic relics found along the Eastern Mediterranean¹ and Egyptian mummies dating back to 2000 BC⁷. Around 460 BC, Hippocrates identified tuberculosis, then known by its Greek name “phthisis,” as the most widespread infectious disease of the age.

It was not until March 24, 1882 that the causative agent of tuberculosis, the bacterium *Mycobacterium tuberculosis*, was identified by Robert Koch. He received the 1905 Nobel Prize for this discovery. In 1890, based on his discovery of tuberculosis, he went on to publish “Koch’s

Postulates”, a list of fundamental principles for identifying disease-causing pathogens⁸. Many of these criteria are still used today in the practice of modern microbiology.

Through the 19th and early 20th century, tuberculosis continued to be a major public health problem fueled by poor urban living conditions that led to high transmission of the disease within crowded city tenements. In the U.S., the rate of death from tuberculosis in 1900 was 194 out of 100,000 people⁹ and tuberculosis was estimated to cause nearly one-quarter of all deaths in Europe (the most by any single cause) through the early 1900’s. Around the same time, a movement in the medical field emerged with doctors moving patients into rural sanatoria, believing that fresh air and rural surroundings would help to cure them.

Despite the length of human history in which public health has battled tuberculosis, there has only been one vaccine, BCG, implemented by public health programs to prevent tuberculosis. BCG is named *Bacillus Calmette–Guérin* after two French doctors at the Pasteur Institute, Albert Calmette and Camille Guérin, who subcultured virulent strains of a related mycobacteria species, *Mycobacterium bovis*, until it lost much of its virulence. It was first used as a vaccine in 1921 and continues to be used today to provide protection for newborns, although its reported efficacy in adults (0-80%) is highly variable¹⁰. The failure of BCG to provide long-lived protection against *Mtb* in adults remains one of the greatest dilemmas in the vaccine development and TB immunity fields. After years of research, it is still not clear what crucial aspect of immunity is missing during BCG-induced immune responses. Many vaccines in the development pipeline seek to boost the BCG response in order to lengthen protection, while others aim to replace it completely. In order to eventually develop an improved vaccine against *Mtb*, much more must be learned about what constitutes an effective immune response.

Immunity to TB

Infection of *Mycobacterium tuberculosis* generally begins with inhalation of aerosolized droplets containing *Mtb* bacilli, coughed up from another infected individual (*Figure 1-1*)¹¹. These droplets fall into distal lung alveoli. While 70% of TB cases are pulmonary, *Mtb* can disseminate

to other organs including lymph node, bones, and the brain¹². Mtb primarily infects alveolar macrophages, but can also infect other cells situated in the lung including dendritic cells¹³, recruited monocyte-derived macrophages¹⁴, neutrophils¹⁵, and epithelial cells¹⁶. As an obligate intracellular bacterium, Mtb has evolved numerous pathways to be taken up by myeloid cells^{17, 18}. Macrophages phagocytose Mtb through a number of different receptors including complement receptors¹⁹, scavenger receptors²⁰, and mannose receptors²¹, many of which are upregulated following recognition of the bacteria through pattern recognition receptors.

Innate Immune recognition of Mtb

Macrophages utilize numerous cell surface and intracellular receptors to recognize Mtb pattern-associated molecular patterns (PAMPs). Surface Toll-like receptor (TLR) TLR2 recognizes lipoproteins, phosphatidylinositol mannans, and lipomannan²², and TLR9 located in phagosomes and lysosomes recognizes Mtb DNA²³. C-type lectin receptors like DC-SIGN and mannose receptor recognize mannose-capped lipoarabinomannan (ManLAM)²⁴, while Dectin-1 recognition of Mtb remains unknown. Mincle was recently shown to recognize trehalose dimycolate (TDM), previously known as mycobacterial cord factor^{25, 26}. Cytosolic receptors like NOD2 and NLRP3 recognize the peptidoglycan subunit N-glycolyl muramyl dipeptide as well as ESX-1 secreted products²⁷.

Many of these receptors have redundant functions and single knock-out murine models generally have minimal phenotypes. However, the lack of certain receptors like NOD2 and the absence of downstream factors that multiple signaling pathways convene on including ASC, caspase-1 and CARD9 lead to diminished immune responses with lower production of pro-inflammatory cytokines like IL-12p70, IL-23, IL-6, IL-1 β and TNF, ultimately leading to decreased T cell activation, higher bacterial burdens, and decreased overall survival of the host^{23, 28, 29, 30, 31}. Interestingly, although mice lacking the signaling factor MyD88 also have increased susceptibility,

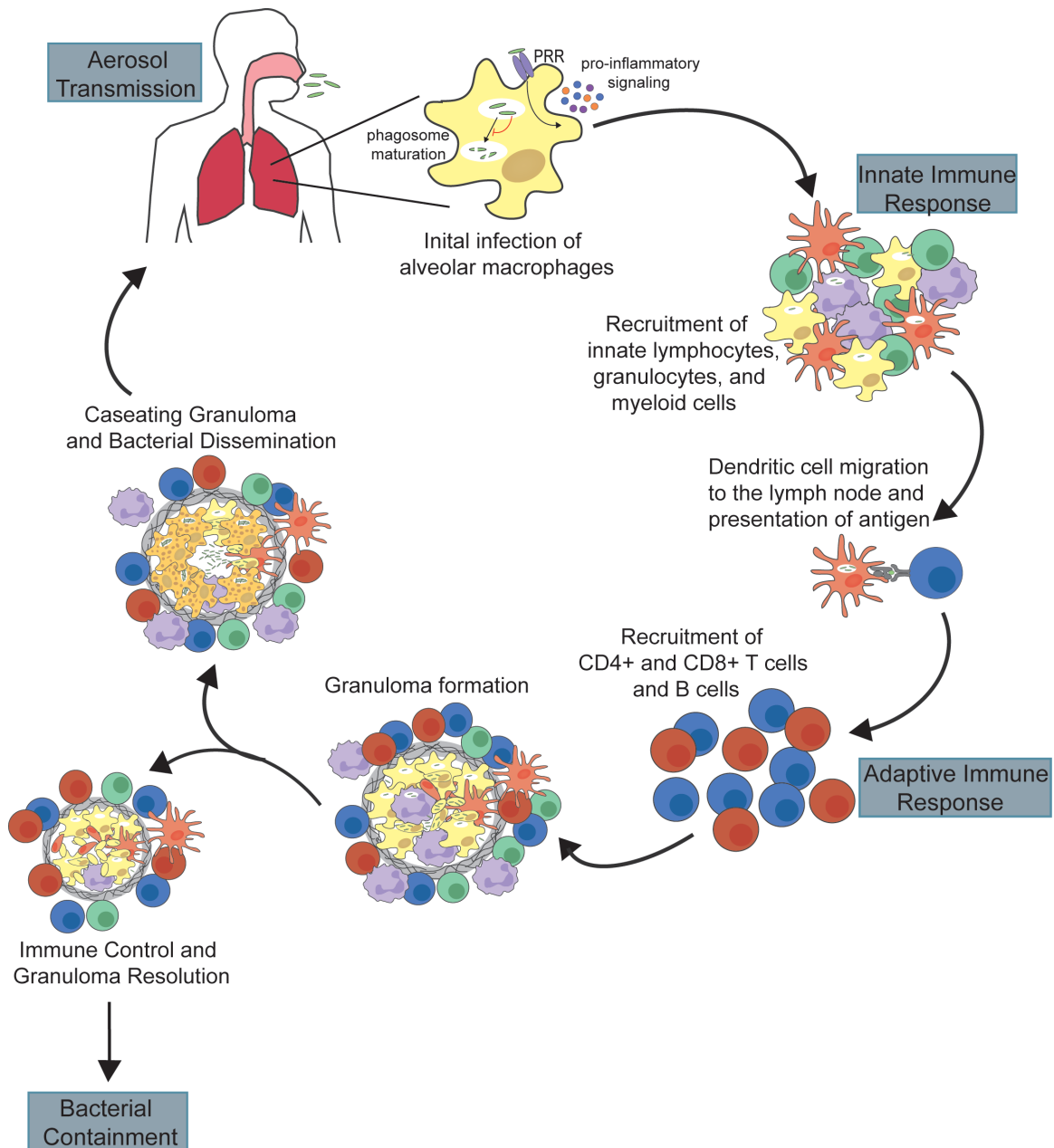


Figure 1-1. Immune Response to *Mycobacterium Tuberculosis*

Mtb infection occurs primarily via the aerosol route, where alveolar macrophages take up the bacteria. Pattern recognition receptors (PRR) detect Mtb and signaling leads to pro-inflammatory cytokine production. During the innate immune response, innate lymphocytes, myeloid cells, and granulocytes are recruited to the lung. After dendritic cell migration to the draining lymph node, the adaptive response is initiated and granulomas are formed. In 90% of infected individuals, the immune response controls the infection leading to bacterial containment. In 10% of individuals, necrosis and caseation leads to bacterial dissemination and further transmission. (Adapted from “In search of a new paradigm for protective immunity to TB” (2014)³²).

this has been attributed to its role in IL-1 β and IL-18 signaling rather than TLR signal transduction^{23, 30, 31, 33}.

Initiation of the adaptive immune response

By day 7 to 10 post-infection, dendritic cells are thought to transport Mtb from the lung to the draining lymph node, where they present antigen to T cells and initiate the adaptive immune response³⁴. Here, IL-12p70 production has been shown to be crucial to generate Th1 IFN γ response³⁵. Antigen-specific T cells expand and differentiate into effector cells, which then migrate back to the lung with other recruited lymphocytes to control the infection (*Figure 1-1*). Knockout mice studies have confirmed the crucial role for CD4⁺ T cells and their production of IFN γ and TNF at this stage^{36, 37, 38, 39}. IFN γ plays an important role in activating infected macrophages to kill intracellular bacteria through expression of inducible nitric oxide synthase (iNOS) leading to production of nitric oxide, IFN γ -inducible GTPases, phagosomal maturation and acidification, Vitamin D receptor signaling and autophagy^{40, 41, 42, 43, 44}. In addition, human genetic studies have linked mutations in IL-12, IFN γ , and STAT1 to disseminated infections of BCG and non-tuberculous mycobacteria (a closely related species to Mtb that is commonly found in the environment)⁴⁵. The presence of T cells in the lung also leads to the formation of granulomas, organized collections of lymphocytes, macrophages, and fibroblasts as well as inflammatory induced cell types including multinucleated giant cells and foam cells designed to wall off and control the growth of bacteria (*Figure 1-1*)⁴⁶. Loss of control of bacterial growth due to T cell exhaustion, immunopathology, and other undescribed conditions leads to recrudescence and reactivation of Mtb. A major focus in the TB field is to better understand the immunological factors that keep Mtb in check and how these may change over the course of chronic infections.

Mtb intracellular survival strategies

Once internalized, Mtb utilizes a number of strategies for intracellular survival. First, Mtb uses a number of secreted virulence factors to inhibit phagosome maturation, preventing

lysosomal fusion and excluding phagosomal vATPase, leading to inhibition of vesicle acidification and cargo degradation normally employed by macrophages to degrade intracellular pathogens^{47, 48, 49}. Second, Mtb expresses an arsenal of enzymes and proteins to detoxify reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) produced by activated macrophages and to repair cellular damage from environmental stresses produced by an active immune response⁵⁰. Third, Mtb induces production of certain lipid mediators like LXA₄ and inhibits the production of others like PGE₂ to alter the cell death modality and inflammatory environment of its host cell^{51, 52}. While a range of cell death fates have been observed during infection, these cellular changes, along with secretion of Mtb virulence factors like nuoG are thought to tip the cell fate of the macrophage in favor of necrosis over apoptosis⁵³. Necrosis leads to release of bacteria and dissemination into other target cells, while apoptosis leads to containment of the bacteria within apoptotic blebs. Efferocytosis, uptake of the apoptotic debris by other macrophages, is associated with ultimate control of bacterial growth^{54, 55, 56}. An apoptotic cell fate has also been linked to more efficient priming of CD8⁺ T cells during the adaptive phase of the immune response⁵⁷. Fourth, Mtb manipulates antigen presentation by downregulating MHC II surface expression, essentially “hiding out” from the CD4⁺ T cell response, once it is initiated⁵⁸.

Critical immune cytokines

Given the many strategies Mtb employs to buffer the antimicrobial efforts of IFN γ -activated macrophages, it is no surprise that other cytokines and cell types also play crucial roles in controlling infection⁵⁹. While CD4⁺ T cell production of IFN γ is critical, research has also shown that it is not sufficient to induce protection. For example, increased risk of infection for HIV-positive patients has reinforced the critical nature of the CD4⁺ T cell response. However, risk for active TB in these patients does not actually correlate with the timing of loss of their CD4⁺ T cells, suggesting that the relationship between CD4⁺ T cells and the immune protection against TB is more complicated^{60, 61}. In addition, a recent vaccine trial using MVA85A, a booster to BCG vaccination that led to enhanced IFN γ and polyfunctional T cell responses in initial studies, failed

to protect 4-6 month infants in a 2-year follow-up study, again suggesting that IFN γ responses are not sufficient to mediate protection⁶². There are now several studies that have directly addressed IFN γ -independent mechanisms of CD4⁺ T cell control^{63, 64}.

There are many other cytokines identified that participate in the immune response against Mtb. IL-18 enhances the IFN γ response as well as plays a secondary antimicrobial role that has yet to be fully worked out⁶⁵. Antimicrobial activity has also been shown for IL-1 α/β ³¹. TNF is another critical T cell effector cytokine during infection and TNF^{-/-} mice have been shown to be quite susceptible to Mtb^{66, 67}. TNF synergizes with IFN γ to induce iNOS production as well as helps to maintain granuloma formation and reduce immunopathology^{52, 68, 69}. The numerous reported cases of reactivated TB upon treatment with anti-TNF blockade for rheumatoid arthritis patients reinforce the importance of TNF in human infection as well⁷⁰.

GM-CSF^{-/-} mice are highly susceptible to Mtb and GM-CSF treatment of human macrophages restricts intracellular growth of Mtb and *M. avium*^{71, 72, 73, 74}. GM-CSF is produced by a multitude of cells including leukocytes, epithelial cells and fibroblasts, and loss of this cytokine leads to abnormalities in surfactant recycling and the development of a lung disease that resembles human pulmonary alveolar proteinosis. Overexpressing GM-CSF in epithelial cells reverses these lung abnormalities but the susceptibility to Mtb remains, suggesting that GM-CSF production by other cells contributes to protection in mice. Additionally, the presence of anti-GM-CSF autoantibodies that block GM-CSF function has been linked to both cryptococcal meningitis and pulmonary TB in otherwise healthy subjects indicating that GM-CSF plays an important role in host defense against infection in people⁷⁵. (The role of GM-CSF during Mtb infection will be discussed and tested further in *Chapter 3* and *4*).

While many pro-inflammatory cytokines have been shown to play positive roles during Mtb infection, murine knock-out studies have shown that mice lacking type I IFN signaling have improved outcomes suggesting that IFN α/β may play detrimental roles during infection, although there is evidence that they can stimulate the immune system and may also have more positive effects too^{76, 77}.

Contribution of additional immune cell types

Murine studies demonstrate a critical role for cell types other than conventional CD4⁺ T cells. In addition, data from non-human primates as well as human studies emphasize how the immune response is most effective when the responses from multiple cell types are coordinated.

Mice and non-human primates lacking CD8⁺ T cells are more susceptible to infection than intact mice, although the phenotype does not appear until very late during infection^{78, 79}. Apart from production of IFN γ , CD8⁺ T cells have been shown to recognize Mtb-antigens and inhibit bacterial growth in vivo via cytolytic activity using perforin and Fas/FasL pathways⁸⁰. Mice that lack these cytolytic effectors are also susceptible and a role for granulysin-mediated killing was demonstrated in human CD8⁺ T cells^{81, 82, 83}. A role for B cells during infection is still unknown⁸⁴.

Th17 CD4⁺ T cells contribute to granuloma formation and recruitment of both antigen-specific Th1 cells and neutrophils to the lung^{85, 86}. IL-23 was shown to be critical for establishing a Th17 response⁸⁷. While a Th17 response contributes to antimicrobial activity early on, adoptive transfer of Th17 cells led to only moderate protection and a prolonged Th17 response has been shown to increase immunopathology^{63, 88}.

Similarly, neutrophils have been shown to play an important antimicrobial role early on during infection by producing pro-inflammatory cytokines and antimicrobial peptides, but their ongoing presence in the lung also leads to excess immunopathology and inflammation that can make resolution of the infection difficult^{89, 90, 91, 92}. A role for IFN γ in limiting neutrophil-mediated inflammation may be an additional underappreciated effect of IFN γ ⁹³. This is one of many immunoregulatory pathways during infection that limit immunopathology. Others include: suppression of IL-1 β by NO⁹⁴, PD-1 expression^{95, 96}, and Foxp3⁺ regulatory T cells^{97, 98, 99}. Although limiting immunopathology is important, the response must be fine-tuned because it comes at the expense of an efficient immune response.

While conventional CD4⁺ and CD8⁺ T cells have been the focus of most vaccine research, there is now renewed interest in reviewing the role of non-classically-restricted T cells during infection¹⁰⁰.

$\gamma\delta$ T cells are not required for bacterial control in mice, yet they are the dominant source of IL-17 during infection^{36, 86}. In a non-human primate model, activation of $\gamma\delta$ T cells by IL-2 and phosphoantigens have been shown to decrease bacterial burden during Mtb infection¹⁰¹. $\gamma\delta$ T cells are capable of forming a recall response after BCG vaccination and Mtb challenge and recently the small organic phosphate antigens and alkylamines recognized by $\gamma\delta$ T cells were identified, opening the door for future investigation of this cell type during Mtb infection^{102, 103, 104}.

Mucosal associated invariant T (MAIT) cells recognize microbial-derived riboflavin metabolites presented on MR1 antigen presenting molecules and, as their name suggests, are found primarily at mucosal tissues, including the lung¹⁰⁵. As innate-like lymphocytes, they share some characteristics with CD1-restricted T cells and $\gamma\delta$ T cells. MAIT cells recognize Mtb-infected cells and have been found in the human lung and peripheral blood¹⁰⁶. While an antimicrobial role has been demonstrated for MAIT cells during other infection models, their role in Mtb infection has yet to be determined¹⁰⁷.

Group I CD1-restricted T cells recognize mycobacterial lipids presented on CD1a, b, or c. While not present in mice, they have been detected in human peripheral blood and Mtb-infected individuals^{108, 109, 110, 111}. CD1a, CD1b and CD1c-restricted T cells specifically recognize mycobacterial lipids: CD1a-restricted T cells recognize dideoxymycobactin, CD1b-restricted T cells recognize glucose monomycolate, and CD1c-restricted T cells recognize C32-phosphomycoketide¹¹². Their function and activation state during infection still remains to be determined.

While there are many cell types whose function during infection requires further investigation, the work of this thesis was initiated by an interest in the role of invariant natural killer T (iNKT) cells during Mtb infection. iNKT cells recognize lipid and glycolipid antigens (*reviewed in depth in the following section*). In human biomarker studies, patients with active TB have reduced iNKT cell numbers in peripheral blood compared to latently infected or healthy individuals^{113, 114, 115}. Murine iNKT cells cultured with Mtb-infected macrophages restrict bacterial growth in vitro and adoptive transfer of iNKT cells limits bacterial growth in vivo¹¹⁶. In addition,

stimulation of infected mice with α GalCer, a potent synthetic ligand and activator of iNKT cells, has been shown to improve disease outcome and synergize with antibiotics^{117, 118}. α GalCer also stimulates human iNKT cells to lyse Mtb-infected macrophages through granulysin production and kill intracellular bacteria in vitro¹¹⁹. This activation has also been implemented as an adjuvant-strategy in vaccine design, where α GalCer was conjugated to BCG to enhance its efficacy¹²⁰. Despite their ability to control Mtb growth when activated, iNKT cells are dispensable for bacterial control in an intact mouse during infection. Whether this is due to a murine-specific reliance on the dominant CD4⁺ T cell pathway, an iNKT cell effector function that is redundant with other cell types, or inhibition of activation during infection will be discussed in this thesis.

Biology of Invariant Natural Killer T (iNKT) cells

NKT cells influence many different immunological conditions including microbial infection, autoimmunity, asthma and allergy, cancer, and graft-versus-host disease¹²¹. NKT cells are thymus-derived T cells that recognize lipid antigens with both CD4⁺ and CD4⁻CD8⁻ double-negative (DN) populations in the mouse and CD4⁺, CD8⁺ or DN populations in humans. They were first discovered in 1987 as TCR $\alpha\beta$ ⁺ DN cells that produced cytokines and later in 1990 as NK1.1⁺ TCR $\alpha\beta$ ⁺ DN cells that were MHC class II-independent¹²². Eventually, these cells were discovered to express a semi-invariant TCR^{123, 124} and to be restricted by CD1d^{125 126}.

The first CD1d-presented lipid antigen to be discovered was α -Galactosylceramide (α GalCer), a lipid derived from marine sponges. Recognition of α GalCer is now used to identify iNKT cell populations¹²⁷.

Group I NKT cells express a semi-invariant TCR with invariant alpha-chains (V α 14-J α 18 in mice and V α 24-J α 18 in humans) paired to a limited set of TCR beta-chains (V β 8.2, V β 7, and V β 2 in mice, and V β 11 in humans) and are the subject of the research in this thesis, further known as invariant NKT (iNKT) cells. Group II NKT cells or diverse NKT (dNKT) cells also recognize lipid antigens but are less prevalent and less well studied due to the challenge of identifying them as a population with diverse TCRs¹²⁸. (*Appendix II* includes one study examining

the recognition of live Mtb infection and isolated mycobacterial lipids by human dNKT cell clones¹²⁹).

While many parallels can be drawn between iNKT cell TCR binding to antigens presented on CD1d and conventional T cell TCR binding to peptide-MHC complexes, there are also striking differences. Crystallography studies have discovered that the binding alignment of the iNKT cell $\alpha\beta$ -TCR with the antigenic groove of CD1d is rotated compared to classical TCR-MHC that binds in a more parallel position¹³⁰. In this configuration, only the CDR3 loop of the TCR α -chain is in contact with the lipid head group, with the CDR3 loop of the TCR β -chain binding directly to CD1d, shedding light on the requirement for α - but not β -chain invariance. This is in contrast to classic TCR-MHC binding where the CDR3 loop of both the α - and β -chain bind the loaded peptide.

CD1d: trafficking, lipid antigen uptake, and presentation

CD1d is one of 5 active CD1 genes found in humans. CD1a, CD1b, and CD1c present antigen to Group I CD1-recognizing T cells¹³¹. While CD1e is not expressed on the cell surface, it is brought directly to endosomal compartments where it may assist in the lipid processing and loading of other CD1 molecules^{132, 133}. Mice have lost CD1a, CD1b, CD1c, and CD1e due to a chromosomal break and express duplicate copies of CD1d¹³⁴, making mice a useful biological tool for studying CD1d and iNKT cells. CD1d has similar structural homology to Class I MHC, with 3 α -domains non-covalently associated with $\beta 2m$ ¹³⁵. CD1d is constitutively expressed by many different cell types including professional antigen presenting cells such as macrophages, dendritic cells, as well as B cells, granulocytes, and epithelial cells^{136, 137}.

CD1d heavy chain is synthesized in the endoplasmic reticulum (ER) and associated with $\beta 2$ -microglobulin ($\beta 2m$). CD1d is first loaded with ER-resident self-lipids with help from microsomal triglyceride transfer protein^{138, 139}. CD1d then follows the secretory route out of the ER, through the Golgi apparatus to the plasma membrane. From the cell surface, CD1d is then endocytosed into endosome and lysosome compartments, where ER-lipids are exchanged for

self- or microbial-lipids with the help of accessory proteins like saposins, apolipoprotein E, and CD1e (in human cells only)^{133, 140, 141, 142}. Interactions with the invariant chain and MHC class II molecules have also been shown to be required for the presentation of lysosomal proteins^{143 144, 145}.

iNKT cell development

iNKT cells develop from the same precursor population as other T lymphocytes, starting in the bone marrow and migrating to the thymus. iNKT cells continue to follow the same pathway of thymic development through the CD4⁺CD8⁺ double-positive stage. Once they express the invariant Vα14 α-chain and limited β-chains through stochastic rearrangement, iNKT cells are positively selected on double-positive cortical thymocytes¹²⁶ through presentation of yet identified self antigens presented on CD1d. Strong TCR signals lead to expression of early growth response protein 1 (EGRP1) and EGRP2, which promote expression of promyelocytic leukemia zinc finger protein (PLZF) transcription factor¹⁴⁶. PLZF is thought to be a master regulator of iNKT cells and its expression is sufficient to promote many of the functions of iNKT cells^{147, 148}. The shared usage of PLZF between iNKT cells and other innate lymphocytes including human MR1-restricted MAIT cells¹⁴⁷ and γδ T cells¹⁴⁹ suggests that PLZF may serve a crucial role in inducing the “innate” phenotype and function shared by these cell types.

iNKT cells then undergo four distinct stages of development, marked by changes in the surface expression of CD24, NK1.1 and CD44: (stage 0) CD24⁺, CD44^{lo}, NK1.1⁻, (1) CD24⁻, CD44^{lo}, NK1.1⁻, (2) CD24⁻, CD44^{hi}, NK1.1⁻, (3) CD24⁻, CD44^{hi}, NK1.1⁺¹²¹. iNKT cells generally exit the thymus at stage 2, where they finish their development in the periphery.

Once in the periphery, iNKT cells undergo final maturation through interactions with professional APCs. While development of iNKT cells does not require CD1d-encoded sorting motifs, the peripheral activation of these cells does, as shown in studies utilizing CD1d-enhanced yellow fluorescent fusion protein (CD1d-EYFP)¹⁵⁰. It has also been recently published that commensal microbiota influence the peripheral development of iNKT cells^{151, 152}.

In C57BL/6J mice, iNKT cells constitute about 0.2-0.5% of total lymphocytes in most organs including spleen, lung, lymph nodes, gastrointestinal tract, thymus, and bone marrow, while they make up between 20-30% of lymphocytes in the liver^{153, 154}. In human peripheral blood, iNKT cells make up 0.1-0.5% of lymphocytes¹⁵⁵. Similar frequencies are found in human spleen, bone marrow, and lymph nodes, and the highest frequencies are found in the liver (1%) and omentum (10%)¹⁵⁶.

iNKT cell activation by TCR-dependent signals

There are just several microbial lipids for iNKT cells that have been identified thus far with reproducible antigenicity. iNKT cells recognize: α -linked glycosphingolipids from *Sphingomonas capsulata*^{157, 158}, α -linked glucosyl or galactosyl diacylglycerol from *Borrelia burgdorferi*¹⁵⁹, and *Streptococcus pneumoniae* (Figure 1-2A)¹⁶⁰.

However, it was noted that there are numerous conditions that stimulate iNKT cells, including viral infections, cancer, autoimmune diseases, as well as developmental requirements for activation that would not occur in the presence of microbial products. Therefore, there was early understanding that self-lipid antigens could play an important role in iNKT cell activation and many groups attempted to identify these antigens. One of the early self-lipids discovered was isoglobotrihexosylceramide (iGb3), although aspects of its antigenicity and role in development are now controversial^{161, 162}. The self antigen lysophosphatidylcholine activates human but not murine iNKT cells¹⁶³. β -glucosylceramide (β GlcCer) was recently shown to activate iNKT cells and accumulate in response to TLR stimulation¹⁶⁴. A role for plasmalogen lysophosphatidylethanolamine (plasmalogen lysoPE) in iNKT cell thymic development was recently demonstrated¹⁶⁵. In addition, a recent study showed that endogenous lipid expression may be regulated by danger signals, where TLR/MyD88 stimulation leads to a decrease in lysosomal α -galactosidase A activity, allowing for the accumulation of stimulatory endogenous lipids¹⁶⁶.

The system is fine-tuned so that every aspect of the loaded lipids contributes to the quality of the response. Lipid acyl chain length alters antigen processing/presentation pathways, while the head group influences affinity and half-life of the interaction¹⁶⁷. Different ligands can induce different response polarization. While initially it was thought that this difference was due to variations in ligand affinities, studies have shown that kinetics of lipid loading and presentation play a larger role than affinity¹⁶⁸. It was recently determined that autoreactivity (recognition of endogenous ligands) could partly be explained by a particular CDR3 β -loop sequence that directly associates with CD1d molecules¹⁶⁹.

iNKT cell activation by TCR-independent signals

In contrast to microbial and endogenous lipid stimulation, iNKT cells can also be activated independently of TCR signals through high concentration of cytokines including: IL-12, IL-18, IL-23, and IL-25 (*Figure 1-2B*)¹⁷⁰. IL-12 especially has been shown to play an important role in iNKT cell activation in response to TLR agonists like LPS¹⁷⁰, CpG and viral infections^{171, 172}.

iNKT cell activation by a combination of TCR-dependent and -independent signals

As studies on iNKT cell activation have progressed, there is more and more evidence that iNKT cells are likely to be most often activated in vivo by a combination of weak TCR-dependent and TCR-independent signals (*Figure 1-2C*). This was first described for *Salmonella typhimurium* infection^{157, 173} and more recently for 11 different heat-killed bacteria strains including gram-positive, gram-negative, and α -proteobacteria, which all required both CD1d APC expression and IL-12p40 for IFN γ production¹⁷⁴. Activation by *Aspergillus fumigatus* requires both CD1d expression as well as IL-12 production by dendritic cells dependent on dectin-1 recognition of β -glucans presented by the fungi¹⁷⁵.

While independent effects of both CD1d-presented lipids and pro-inflammatory cytokines have been demonstrated for iNKT cells, there are also additional links in signaling that have been defined between these pathways. There are numerous examples of cytokine signals inducing

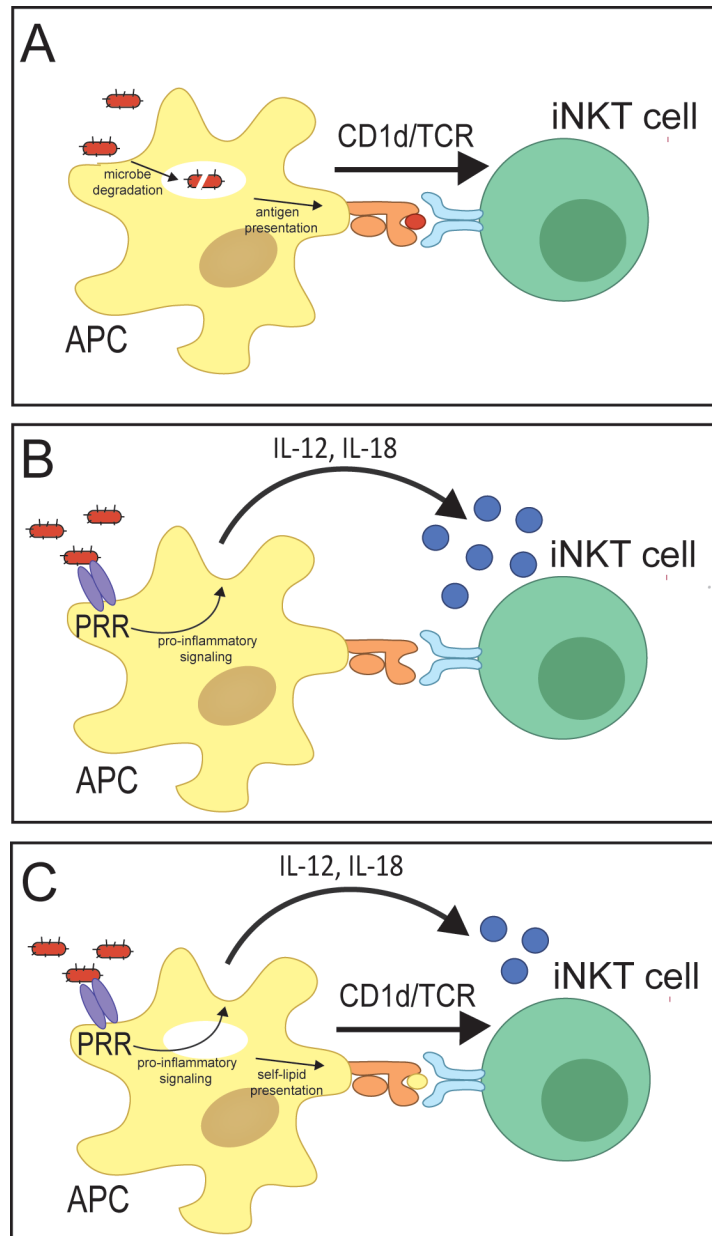


Figure 1-2. Activation pathways of iNKT cells

iNKT cells are activated during infection by multiple pathways. (A) TCR-dependent pathway. iNKT cells are activated by CD1d-mediated presentation of microbial lipids through their TCR. (B) TCR-independent pathway. iNKT cells are activated by strong pro-inflammatory cytokines like IL-12 and IL-18. (C) Combination TCR-dependent and -independent pathway. iNKT cells are activated by a combination of weak self-lipid presentation and low pro-inflammatory cytokine production.

changes in CD1d expression on APCs. Type I IFN was reported to upregulate CD1d expression during *Listeria monocytogenes* infection¹⁷⁶. IFN γ and TNF produced by mycobacterial products induces CD1d expression on macrophages during Mtb infection¹⁷⁷. In addition, a panel of TLR agonists leads to changes in glycosphingolipid biosynthesis in dendritic cells and increases in IFN γ production by co-cultured iNKT cells¹⁷⁸.

iNKT cell effector functions

iNKT cells have been coined “the Swiss-Army knife of the immune system”¹⁷⁹ due to their diverse repertoire of effector functions (*Figure 1-3*). They are best known for their ability to rapidly produce cytokines. These include: IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17, IL-21, IFN γ , GM-CSF, TNF, TGF- β and chemokines RANTES, Eotaxin, LT, MIP-1 α , and MIP-1 β ^{179, 180}. iNKT cells also have demonstrated cytolytic ability. In several murine studies, in vitro and in vivo α GalCer stimulation induced cytotoxicity that was predominantly Fas/FasL dependent^{181, 182}. α GalCer stimulation of human iNKT cell clones also induced granulysin-mediated cytotoxicity of Mtb-infected macrophages¹¹⁹.

Given the many effector functions ascribed to iNKT cells, many studies have divided iNKT cell populations into various subsets based on their function. One study of human peripheral iNKT cells found that effector function could be grouped by CD4 expression¹⁵⁵. CD4⁻ iNKT cells predominantly produced Th1 cytokines IFN γ and TNF, while CD4⁺ iNKT cells produced both Th1 cytokines as well as Th2 cytokines like IL-4, IL-5, and IL-13. Apart from “Th1” and “Th2” iNKT cell subsets, several other minor populations have been described. Th17 iNKT cells require ROR γ t for development in the thymus, produce IL-17A, respond to IL-23, and are involved in neutrophil recruitment in the lung in mice, although a resident population of human Th17 iNKT cells has not been verified yet^{183, 184}. An IL-21 producing follicular helper (Tfh) iNKT cell subset and an in vitro derived Foxp3⁺ regulatory T cell (Treg) iNKT subset have also been described^{185, 186}.

Because iNKT cells respond so rapidly and to many different inflammatory signals, studies have recognized their enormous influence in altering other aspects of the immune system.

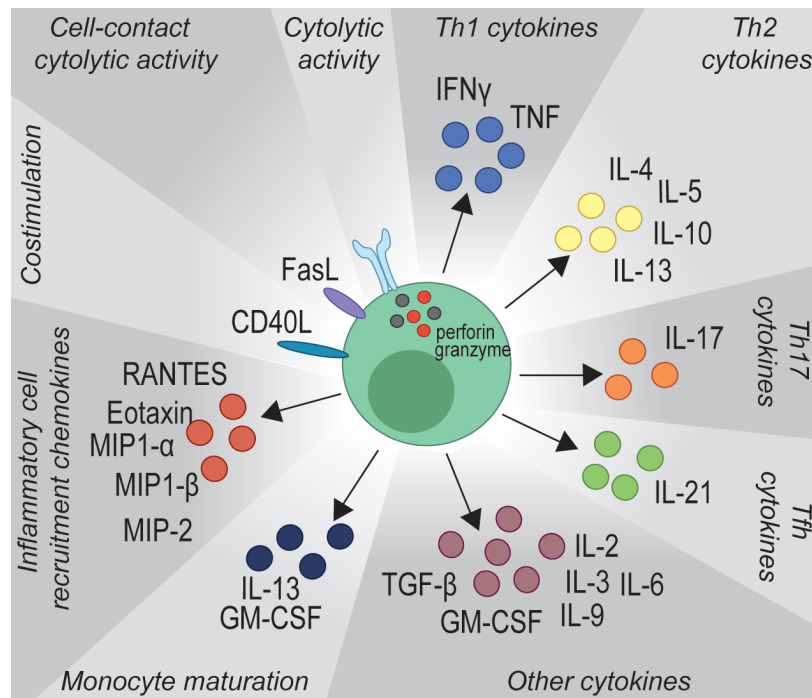


Figure 1-3. iNKT cell effector functions

iNKT cells have multiple effector functions including: production of Th1, Th2, and Th17 cytokines, secretion of factors for monocyte maturation, chemokine production for recruitment of inflammatory cells, costimulation, and cytolytic activity.

iNKT-mediated immunomodulation can have dramatic pro-inflammatory effects. Through many mechanisms, iNKT cells can influence the activation of dendritic cells. Production of GM-CSF and IL-13 by iNKT cells leads to the differentiation of monocytes into dendritic cells¹⁸⁷. Early iNKT cell IFN γ production as well as costimulation through CD40/CD40L lead to maturation of DCs and increased IL-12 production from DCs that subsequently enhances CD8⁺ T cell cross-priming^{188, 189}. iNKT cells can also activate macrophages in a similar manner¹⁹⁰. iNKT cell IFN γ production can also lead to trans-activation of NK cells¹⁹¹. iNKT cells also provide cognate B cell help¹⁹². Tim-1 expression on iNKT cells leads to activation by dying cells expressing phosphatidylserine, and this recognition was shown to contribute to airway inflammation¹⁹³.

Under certain conditions, immunomodulation by iNKT cells can also lead to immunosuppression. A number of studies have identified iNKT cells as an intermediary in the downregulating effect of type I IFN on CD8⁺ T cell priming in adjuvant stimulation and multiple sclerosis therapy^{194, 195}. A lung inflammation model of IL-33-induced inflammation showed that iNKT cells led to downregulation of pulmonary inflammation, where J α 18^{-/-} mice had increased inflammation and neutrophil recruitment¹⁹⁶. Similarly, an anti-inflammatory role has been attributed to iNKT cells in the lung and liver during the resolving stages of infection with BCG (*M. bovis*)¹⁹⁷. A number of studies have found a role for iNKT cells during airway inflammation in response to environmental antigens¹⁹⁸, airborne lipid antigens¹⁹⁹, susceptibility to acute influenza A²⁰⁰, as well as human asthma and COPD studies²⁰¹.

Role for iNKT cells during microbial infection

The majority of the research on iNKT cells and infection has focused on the activating signals of iNKT cells. As a result, the number of infectious agents that have been demonstrated to activate iNKT cells is greater than the number of diseases where iNKT cells have been shown to be required. In addition, the majority of the studies demonstrating that iNKT cells are essential for pathogen control used knock-out animals to establish the requirement for iNKT cells, while very few studies followed up to determine the specific effector function utilized by iNKT cells to achieve

control. This section aims to summarize the studies thus far that have established a role for iNKT cells during infection and emphasize known effector functions.

αGalCer-induced iNKT cell antimicrobial activity

One of the easiest ways to assess the function of iNKT cells during infection is to treat infected animals with αGalCer, an agent known to selectively activate and expand iNKT cells. Using this strategy, improved disease outcome has been observed after αGalCer treatment in bacterial, viral, parasite, and fungal infection models (*See Table 1-1 for a full list and citations*). In almost all studies that measured changes in iNKT cell function after αGalCer treatment, IFN γ was the effector function that was measured and the effector function that was found to be required. The other main immunological change observed in these studies was an expansion of CD8 $^{+}$ T cells, although this could also be attributed to an increase in IFN γ . In very few studies was any other effector function measured, despite the fact that αGalCer treatment has been linked to production of many different cytokines¹⁸⁰ as well as cytolytic activity^{181, 182}.

Use of murine knock-out models to assess requirement for iNKT cells during infection

Once murine knock-out models like CD1d $^{-/-}$ and Jα18 $^{-/-}$ became available to study infection in the absence of iNKT cells, this strategy became the easiest way to assess the role of iNKT cells. Additionally, comparing the two knock-out models allowed the role of diverse NKT cells to also be determined. There are at least 10 bacterial infections, 5 parasite models, 6 viral infections, and 1 fungus infection model that have associated the presence of iNKT cells with improved disease outcome and just 3 bacterial infections that demonstrated a worse outcome in the presence of iNKT cells (*see Tables 1-2, 1-3, 1-4 for study details and citations*)²⁰².

The three infections where iNKT cells were demonstrated to have a negative impact on disease outcome reveal important aspects of iNKT biology. iNKT cells worsened infection of one strain of Chlamydia, (*C. muridarum*, the murine model for *C. trachomatis*) but not another (*C. pneumoniae*)^{203, 204}. In these studies, the different strains polarized the immune response to either

Table1-1. α GalCer-induced iNKT cell antimicrobial activity

Pathogen strain	α GalCer treatment	Disease outcome	iNKT cell effector function	Function shown to control infection	Citation
<i>Brucella suis</i>	α GalCer loaded macrophages in vitro	Decreased CFU	IFN γ , cytotoxicity	IFN γ , Fas/FasL, perforin	205
<i>Chlamydia pneumoniae</i>	ip injection	Decreased CFU	IFN γ , decreased IL-4		204
<i>Listeria monocytogenes</i>	ip injection	Decreased CFU	IFN γ , NO	IFN γ	206
<i>Mycobacterium tuberculosis</i>	ip injection	Decreased CFU			118
	ip injection, with antibiotics	Decreased CFU, synergistic with antibiotics	IFN γ		117
	vaccination, added to BCG	Decreased CFU after challenge	Increased CD8 ⁺ T cell response		120
	α GalCer loaded macrophages in vitro	Decreased CFU	granulysin-mediated cytotoxicity	granulysin	119
<i>Pseudomonas aeruginosa</i>	ip injection	Decreased CFU	IFN γ		207
	ip injection	Decreased CFU	IFN γ , TNF		208
EMCV-D	ip injection	Decreased paralysis			209
HBV	iv injection	Decreased viral load	Increased IFN α , IFN β , IFN γ	IFN α , IFN β , IFN γ (not shown to be direct)	210
Influenza	ip injection	Decreased viral load, increased survival			211
RSV	ip injection	Delayed viral clearance, decreased immunopathology	Enhanced CD8 ⁺ T cell response		212
<i>Cryptococcus neoformans</i>	ip injection	Decreased CFU	IFN γ	IFN γ	213
<i>Plasmodium berghei</i>	ip injection	Decreased parasite burden	IFN γ	IFN γ	214
	Vaccine, added to irradiated sporozoites or recombinant virus	Decreased parasite burden	IFN γ	IFN γ	215
<i>Trypanosoma cruzi</i>	ip injection	Decreased parasite burden, increased survival			216

a Th1 IFN γ (*C. pneumoniae*) or Th2 IL-4 response (*C. muridarum*). This polarization was exacerbated by the presence of iNKT cells and further by the stimulation of iNKT cells with α GalCer. Here, the strain that induced iNKT cell Th2 polarization, but not Th1 polarization, led to a worsening of disease in the presence of these cells. This result suggests that iNKT cell polarization and the subsequent transactivation of other cell types may be a critical component of iNKT cell effector responses during infection.

In the second infection, the presence of iNKT cells was critical for bacterial control with *Sphingomonas capsulata*, yet mice lacking iNKT cells were spared from fatal septic shock induced by high dose iv infection¹⁵⁷. These results suggest that the rapid and strong response produced by iNKT cells is generally beneficial to the host, but under conditions of massive inflammation, iNKT cells can push the system over the edge.

In the third infection model, mice infected with an α -proteobacteria strain commonly found in soil and water, *Novosphingobium aromaticivorans*, developed autoantibodies against a mitochondrial enzyme leading to liver damage similar to human primary biliary cirrhosis²¹⁷. This autoimmune effect was mediated by iNKT cells and mixed bone marrow chimera data suggest iNKT cells may provide direct B cell help to induce autoantibody production through CD1d-mediated recognition. Other data suggest that the biological link between mitochondrial Pyruvate Dehydrogenase Complex E2 (PDC-E2) and *Novosphingobium aromaticivorans* may be an example of molecular mimicry.

Pathogen-induced iNKT cell antimicrobial activity

The majority of studies examining iNKT cell antimicrobial activity measured iNKT cell IFN γ production during infection. One study of *Streptococcus pneumoniae* infection found that J α 18^{-/-} mice had increased susceptibility compared to WT mice²¹⁸ and treatment with recombinant IFN γ reversed this phenotype. Adoptively transferring liver mononuclear cells, as a source of primary iNKT cells, from both WT and IFN γ ^{-/-} mice into J α 18^{-/-} hosts, the authors demonstrated that IFN γ directly coming from iNKT cells was required for their effect. However, it was not clear

Table 1-2. iNKT cell effector function during bacterial infection.

Pathogen strain	KO model	Disease outcome	iNKT cell effector function	Function shown to control infection	Citation
<i>Borrelia burgdorferi</i>	J α 18 ^{-/-}	Increased CFU	IFN γ		219
	CD1d ^{-/-}	Increased CFU	IFN γ		220
<i>Chlamydia pneumoniae</i>	J α 18 ^{-/-} , CD1d ^{-/-}	Increased CFU, Increased immunopathology	IFN γ , increase in T cell IFN γ		203
<i>Ehrlichia muris</i>	CD1d ^{-/-}	Increased CFU	IFN γ		157
<i>Listeria monocytogenes</i>	CD1d ^{-/-}	Increased CFU	IFN γ		221
<i>Mycobacterium bovis</i>	J α 18 ^{-/-}	Increased immunopathology	anti-inflammatory		197
<i>Mycobacterium tuberculosis</i>	Anti-CD1d	Increased CFU (early)			222
	In vitro infection, adoptive transfer	Addition of iNKT cells decreased CFU in vitro and in vivo	IFN γ		116
<i>Pseudomonas aeruginosa</i>	CD1d ^{-/-}	Increased CFU	PMN recruitment, MIP-2		207
<i>Sphingomonas capsulata</i>	J α 18 ^{-/-} , CD1d ^{-/-}	Increased CFU	IFN γ		157
<i>Sphingomonas yanoikuyae</i>	CD1d ^{-/-}	Increased CFU	IFN γ , IL-4		158
<i>Streptococcus pneumoniae</i>	J α 18 ^{-/-}	Increased CFU, decreased survival	IFN γ		174
	J α 18 ^{-/-}	Increased CFU, decreased survival	PMN recruitment		223
	J α 18 ^{-/-}	Decreased survival	IFN γ	IFN γ	218

Table 1-3. iNKT cell effector function during viral infection.

Pathogen strain	KO model	Disease outcome	iNKT cell effector function	Function shown to control infection	Citation
EMCV-D	CD1d ^{-/-}	Increased paralysis	IFN γ		224
	CD1d ^{-/-} , in vitro infection	Increased viral titer in vivo and in vitro	Enhanced T cell activation		225
	CD1d ^{-/-}	Increased paralysis			209
HSV-1	J α 18 ^{-/-} , CD1d ^{-/-}	Increased viral load			226
	J α 18 ^{-/-} , CD1d ^{-/-}	Increased viral load, decreased survival (CD1d ^{-/-} only)	IFN γ		227
HSV-2	CD1d ^{-/-}	Decreased survival	Early IFN γ		228
Influenza A	J α 18 ^{-/-} , CD1d ^{-/-}	Decreased survival	CD40/CD40L; Inhibition of MDSCs and expansion of CD8 ⁺ T cells		229
		Decrease in epithelial cell death	IL-22		230
	J α 18 ^{-/-}	Decreased survival, no change in viral load	Inhibition of inflammatory monocytes		231
	J α 18 ^{-/-}	Decreased survival, increased pathology, no change in viral load	Expansion of antigen-specific CD8 ⁺ T cells, increase in CD103 DC		200
MCMV	CD1d ^{-/-}	Decreased survival	IFN γ , increase in NK cell IFN γ		232
RSV	CD1d ^{-/-}	Increased viral load	Expansion of antigen-specific CD8 ⁺ T cells		212

Table 1-4. iNKT cell effector function during parasitic and fungal infections.

Pathogen strain	KO model	Disease outcome	iNKT cell effector function	Function shown to control infection	Citation
<i>Aspergillus fumigatus</i>	CD1d ^{-/-}	Increased CFU	IFN γ		175
<i>Leishmania major</i>	J α 18 ^{-/-}	Increased parasite burden (iv, not sc)	IFN γ , enhanced NK cytotoxicity		233
<i>Leishmania donovani</i>	CD1d ^{-/-}	Increased parasite burden	IFN γ		234
<i>Plasmodium berghei</i>	CD1d ^{-/-}	Decreased survival (BALB/c, not B6)	Th2 polarization		235
<i>Trypanosoma cruzi</i>	Anti-CD1d	Decreased survival			236
	J α 18 ^{-/-} , CD1d ^{-/-}	Decreased survival (J α 18 ^{-/-} only)	Decreased cytokine production		237
	J α 18 ^{-/-} , CD1d ^{-/-}	Increased parasite burden	IFN γ , IL-4		238
<i>Toxoplasma gondii</i>	J α 18 ^{-/-} , CD1d ^{-/-}	Decreased survival, increased immunopathology, no change in parasite burden	Decreased CD4 ⁺ T cell activation		239

whether in this model, the IFN γ had a direct antimicrobial effect or transactivated other immune cell types.

Another function attributed to iNKT cells during infection is the expansion of CD8⁺ T cell responses^{200, 212, 225, 229}. De Santo et al found during Influenza A viral infection, in a CD40/CD40L-dependent mechanism iNKT cells inhibited a population of myeloid derived suppressor cells (MDSC) which allowed the expansion of CD8⁺ T cells²²⁹. In another Influenza A model, iNKT cell-mediated expansion of CD103⁺ dendritic cells increased CD8⁺ T cell responses²⁰⁰.

Other attributed effector functions during infections include: neutrophil recruitment^{207, 223}, IL-22 production²³⁰, anti-inflammatory effects^{197, 231, 240}, transactivation of NK cells both for increased IFN γ production²³², as well as enhanced cytolytic activity²³³.

Recently, using spinning disk confocal intravital microscopy, Lee et al visualized hepatic iNKT cells responding to *Borrelia burgdorferi* infection²²⁰. The authors found that iNKT cells interacted with Kupffer cells, after responding to CXCR3 signals. The activated iNKT cells produced IFN γ and clustered in stable CD1d-dependent contacts with the Kupffer cells. These cell contact interactions were strong enough to lead to a dramatic arrest of iNKT cell movement in the liver.

While most studies have focused on the activation and function of iNKT cells during infection, there are also indications that after their rapid responses, iNKT cells can become hyporesponsive and undergo cell death and contraction before the infection has been resolved.²⁴¹

While iNKT cells have been shown to have important roles in many different immunological conditions including infection, cancer, autoimmunity, and graft-versus-host disease, the full spectrum of their effector functions in each of these models has yet to be catalogued. Given the new tools available to iNKT cell researchers, including multicolor flow cytometry, microarray analysis, and multiplex immunoassays, multiple effector functions will now be able to be assayed at one time, allowing for a more nuanced and comprehensive view of iNKT cell function.

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Chapter 2: iNKT cell production of GM-CSF controls *Mycobacterium tuberculosis*

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ABSTRACT

Invariant natural killer T (iNKT) cells are activated during infection, but how they limit microbial growth is unknown in most cases. We investigated how iNKT cells suppress intracellular *Mycobacterium tuberculosis* (Mtb) replication. When co-cultured with infected macrophages, iNKT cell activation, as measured by CD25 upregulation and IFN γ production, was primarily driven by IL-12 and IL-18. In contrast, iNKT cell control of Mtb growth was CD1d-dependent, and did not require IL-12, IL-18, or IFN γ . This demonstrated that conventional activation markers did not correlate with iNKT cell effector function during Mtb infection. iNKT cell control of Mtb replication was also independent of TNF and cell-mediated cytotoxicity. By dissociating cytokine-driven activation and CD1d-restricted effector function, we uncovered a novel mediator of iNKT cell antimicrobial activity: GM-CSF. iNKT cells produced GM-CSF in vitro and in vivo in a CD1d-dependent manner during Mtb infection, and GM-CSF was both necessary and sufficient to control Mtb growth. Here, we have identified GM-CSF production as a novel iNKT cell antimicrobial effector function and uncovered a potential role for GM-CSF in T cell immunity against Mtb.

INTRODUCTION

CD1 restricted T cells were first proposed to have a role in antimicrobial immunity based on the observations that CD4⁺CD8⁻ (DN) T cells restricted by group 1 CD1 (CD1a, CD1b, and CD1c) recognized unique and complex lipids from the Mtb cell wall^{1, 2}. Similarly, invariant natural killer T (iNKT) cell antimicrobial function was originally based on the recognition of microbial lipid or glycolipid molecules presented by the MHC-like molecule CD1d. iNKT cells are now recognized to influence many different immunological conditions including autoimmune disease, asthma and allergy, anti-tumor response, graft-versus-host disease, and infection³.

There are several pathways by which iNKT cells can be activated. Classically, high affinity antigens that are potent agonists, typified by the synthetic lipid α -galactosylceramide (α GalCer), trigger TCR activation in a CD1d-dependent manner. Several infectious agents produce microbial lipids that are presented on CD1d and recognized by iNKT cells including *Borrelia burgdorferi* and *Sphingomonas capsulata*^{4, 5, 6}. Activation can also occur when iNKT cells recognize a weak lower affinity self or microbial ligand, insufficient by itself to induce activation, in the context of costimulatory signals. This mode of activation has been shown for pathogens, such as *Salmonella typhimurium*, viruses such as *Influenza A*, and fungi such as *Aspergillus fumigatus*^{7, 8, 9}. There is strong evidence that a major driver of this type of iNKT cell activation is IL-12, which is produced when microbial danger signals stimulate pattern recognition receptors such as Toll-like receptors (TLRs) or dectin-1^{7, 9, 10}.

One of the remaining unanswered questions in iNKT cell biology is what specific role these cells have during infection and whether their activation by different pathways leads to the expression of diverse functions. To address the role of iNKT cells during infection, many of these studies have used mouse models that lack iNKT cells (Ja18^{-/-}, CD1d^{-/-}) or administered α GalCer, a potent activator of iNKT cells. While this strategy has been useful for determining whether iNKT cells are required for host resistance, and for revealing potential antimicrobial effector functions induced after strong activation, much less is known about the physiological role of iNKT cells during infection. Tracking iNKT cell function in vivo has relied extensively on CD69 upregulation

and IFN γ production. Interestingly, in the absence of exogenous α GalCer treatment, there is little evidence that IFN γ production by iNKT cells is protective during infection¹¹. Given that iNKT cells are capable of producing a variety of different cytokines and chemokines including IL-4, IL-10, IL-17, TNF, GM-CSF, MIP-1 α , MIP-1 β as well as having immunomodulatory effects via expression of CD40 and other costimulatory ligands¹², it is surprising that it is still unknown for most infections whether iNKT cells have a direct antimicrobial role. Only in select cases have protective mechanisms been defined. For example, iNKT cells are important for neutrophil recruitment to the lung during *Pseudomonas* infection¹³ and IFN γ is required for protection by iNKT cells against *Streptococcus pneumoniae*, although this could represent a direct or indirect effect¹¹.

There are several lines of evidence that activated iNKT cells enhance host resistance to Mtb. Administration of α GalCer, which activates iNKT cells in vivo, significantly prolongs the survival of susceptible mouse strains following Mtb infection and this effect is synergistic with antibiotics^{14, 15}. α GalCer activates human iNKT cells to lyse Mtb-infected macrophages and kill intracellular bacteria¹⁶. Even BCG vaccination is more effective when it is conjugated with α GalCer¹⁷. Although α GalCer is used as a pharmacological activator of iNKT cells in this context, it is not required. iNKT cells cultured with Mtb-infected primary macrophages stimulate antimicrobial activity that restricts bacterial growth and adoptive transfer of iNKT cells limits bacterial growth in vivo¹⁸. Finally, several clinical studies find that a decrease of iNKT cells in the periphery is a marker of active disease compared to latent infection or healthy controls^{19, 20, 21}. Despite these findings of iNKT cell activation leading to enhanced control, iNKT cells are dispensable in the murine model of chronic tuberculosis infection^{22, 23, 24, 25}.

Our model, in which iNKT cells activate infected macrophages to control Mtb infection in the absence of exogenous stimulation (e.g., α GalCer), provided a unique opportunity to define the direct effector functions of iNKT cells. This model allowed us to study the interaction between iNKT cells and Mtb-infected macrophages and provided the opportunity to perturb specific signaling and effector pathways and then measure subsequent changes in bacterial control. Greater insight into the effector function of iNKT cells during Mtb infection could lead to novel

therapeutic approaches for augmenting their antimicrobial capacity and boosting the host immune response during infection^{14, 15}.

Here we report that iNKT cells upregulated activation markers and produced IFN γ during Mtb infection in vitro and these markers of activation were driven largely by IL-12 and IL-18. Blocking these cytokine signals almost completely inhibited IFN γ production, but surprisingly had little effect on the ability of iNKT cells to control Mtb growth. In contrast, the antimicrobial function of iNKT cells required CD1d signaling and was mediated by a soluble factor, GM-CSF. Blocking GM-CSF abrogated restriction of bacterial growth by iNKT cells and GM-CSF was sufficient to inhibit mycobacterial growth in vitro. We identified GM-CSF as an antimycobacterial effector molecule produced by iNKT cells with the ability to suppress Mtb infection.

RESULTS

iNKT cells are activated by Mtb-infected macrophages

CD69 and CD25, both classic T cell activation markers, as well as IFN γ production, were chosen to track the activation of iNKT cells by Mtb-infected macrophages. Primary mouse iNKT cell lines were co-cultured with thioglycollate-elicited peritoneal macrophages infected with increasing multiplicity of infection (MOI) of H37Rv, a virulent strain of Mtb. After 24 hours, iNKT cells cultured with Mtb-infected macrophages expressed higher levels of CD69 and CD25 and produced more IFN γ compared to iNKT cells cultured with uninfected macrophages (*Figure 2-1*). A similar activation pattern was observed when hepatic mononuclear cells (HMNC), a source of primary uncultured iNKT cells, were used (*Figure 2-2A*) or when iNKT cells were cultured with H37Rv-infected bone marrow derived macrophages (BMDM) (*Figure 2-2B*). These data indicate that iNKT cells become activated after stimulation with Mtb-infected APCs.

iNKT cell activation by Mtb-infected macrophages requires a combination of cytokine and CD1d-dependent signals

Infection by various microbes including *Salmonella*, *Aspergillus*, and *E. coli* LPS, induce iNKT cell activation by a combination of IL-12, IL-18, and/or TCR stimulation through interaction with CD1d^{7, 9, 10, 26}. To determine whether these signals were required for the activation of iNKT cells by Mtb-infected macrophages, we added neutralizing antibodies to cell co-cultures and measured iNKT cell activation after 24 hours. We found that CD25 and IFN γ were inhibited to varying degrees by blockade of the activating signals (*Figures 2-3A and 2-3B*). CD69 expression was more variable and blocking antibodies had little effect on its expression (data not shown). Blocking cytokine signals (IL-12p40, IL-18) had a greater inhibitory effect on the markers than blocking the TCR-CD1d interaction. For example, anti-IL-12p40 reduced CD25 surface expression by $45.7 \pm 3.3\%$, and inhibited IFN γ production nearly completely ($91.4 \pm 4.4\%$) (mean \pm SEM, n=4 experiments) (*Figures 2-3A and 2-3B*). In contrast, anti-CD1d had no effect on CD25 expression and only a modest effect on IFN γ production ($37.5 \pm 6.4\%$ reduction) (mean \pm SEM,

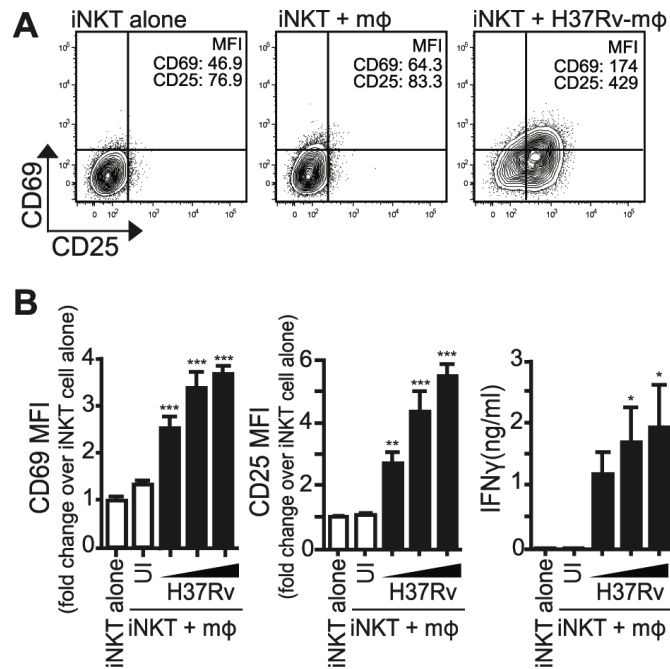


Figure 2-1. iNKT cells are activated by Mtb-infected mφ.

(A) iNKT cells were cultured either alone, with uninfected thioglycollate-elicited peritoneal mφ, or H37Rv-infected mφ for 24 hours. Cells were stained for CD69 and CD25 and mφ were distinguished from iNKT cells by F4/80 staining. (B) Fold change in CD69 and CD25 MFI on iNKT cells cultured with uninfected or H37Rv-infected mφ compared to iNKT cells alone. Supernatant was harvested at 24 hours and IFNγ measured by ELISA. Error bars indicate mean ± SEM. *P < .05, **P < .01, ***P < .001. (One-way ANOVA with Dunnett's post-test, compared to iNKT cells alone). Data are representative of eight independent experiments. Mφ, macrophage; UI, uninfected; ▲, MOI titration, 1.5:1, 3:1, 6:1.

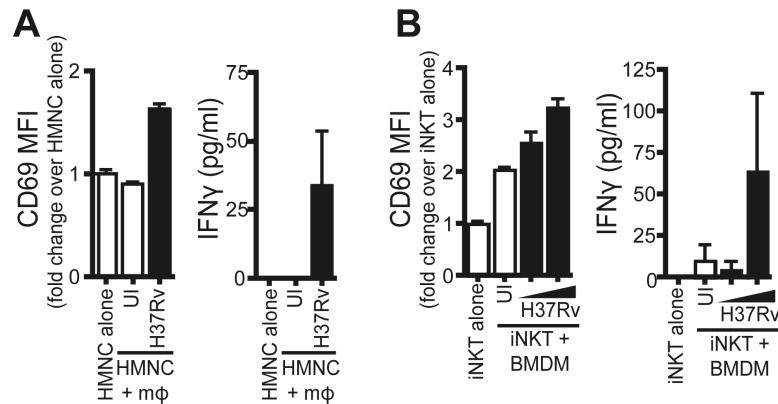


Figure 2-2. iNKT cell activation after Mtb infection is observed with primary iNKT cells and bone marrow derived-macrophages (BMDM).

(A) HMNC were cultured either alone, with uninfected thioglycollate-elicited peritoneal mφ (TGL-PM), or H37Rv-infected TGL-PM for 24 hours and gated on CD1d-tetramer⁺CD3⁺ population. (B) iNKT cells were cultured with uninfected or H37Rv-infected WT bone marrow derived macrophages (BMDM) for 24 hours. Fold change in CD69 MFI over HMNC (A) and iNKT cells (B) cultured alone. Supernatant was harvested at 24 hours and IFNγ measured by ELISA. Error bars indicate mean ± SEM. The data are from single experiments.

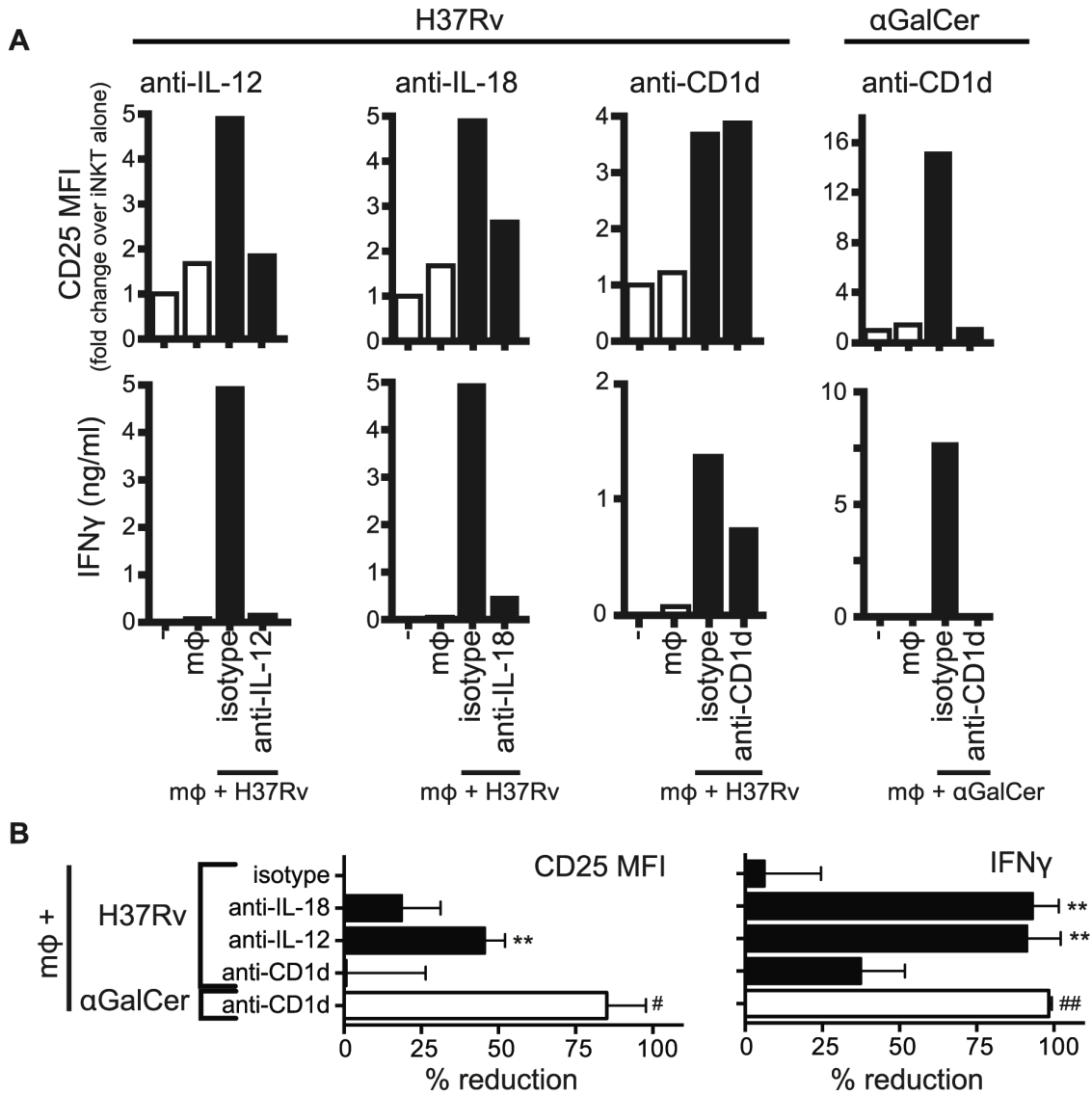


Figure 2-3. Production of IL-12 and IL-18 by Mtb-infected mφ induce traditional markers of iNKT cell activation.

(A) iNKT cells were cultured either alone, with uninfected mφ, H37Rv-infected mφ, or αGalCer-loaded mφ for 24 hours in the presence of blocking antibodies against IL-12p40 (20 μg/ml), IL-18 (10 μg/ml), CD1d (20 μg/ml), or respective isotype controls. Cells were stained for CD25 and mφ were distinguished from iNKT cells by F4/80 staining. Supernatant was harvested at 24 hours and IFNγ measured by ELISA. (B) % reduction calculated as $100 \times [(iNKT_{H37Rv-m\phi} - iNKT_{alone}) - (iNKT_{Ab+H37Rv-m\phi} - iNKT_{alone})] / (iNKT_{H37Rv-m\phi} - iNKT_{alone})$. Conditions with αGalCer stimulation calculated similarly. Supernatant was harvested at 24 hours and IFNγ and IL-12p40 measured by ELISA. Error bars indicate mean ± SEM. **P < 0.01 compared to isotype control. (One-way ANOVA with Dunnett's post-test). #P < 0.05, ##P < 0.01 compared to isotype control (data not shown) (unpaired Student's t-test). Data are representative of, or compiled from three (anti-CD1d), four (anti-IL-12), or two (anti-IL-18) independent experiments.

n=3-4 experiments). The failure of anti-CD1d to block iNKT cell activation was not due to a problem with the experimental conditions since anti-CD1d blocked induction of CD25 and abrogated IFN γ production after α -GalCer stimulation of iNKT cells (*Figures 2-3A and 2-3B*). To verify that cytokines were driving iNKT cell activation, iNKT cells were cultured with MyD88^{-/-} macrophages, which do not produce IL-12 after H37Rv infection²⁷ (*Figure 2-4*). When co-cultured with Mtb-infected MyD88^{-/-} macrophages, iNKT cells did not upregulate CD25 or secrete IFN γ . These results indicated that IL-12 and IL-18 produced by macrophages during Mtb infection drove the activation of iNKT cells as measured by induction of CD25 surface expression and IFN γ production. Thus, these conventional markers of iNKT cell activation were not reliable indicators of CD1d-dependent TCR-mediated signaling during Mtb infection.

Control of intracellular Mtb growth by iNKT cells requires CD1d but not IL-12 and IL-18

As shown previously, iNKT cells cultured with H37Rv-infected macrophages reduced intracellular bacterial growth over a 5 day infection¹⁸ (*Figure 2-5A*). We used this co-culture model to evaluate how the different activation signals affected iNKT cell antimicrobial activity.

We first tested the requirement for IL-12 and IL-18 signaling. The addition of anti-IL-12p40 and/or anti-IL-18 blocking antibodies to co-cultures of iNKT cells and H37Rv-infected WT macrophages did not affect the ability of iNKT cells to inhibit bacterial growth (*Figure 2-5A*). We confirmed this result using MyD88^{-/-} macrophages, which did not induce upregulation of CD25 or IFN γ by iNKT cells (*Figure 2-4*). iNKT cells were able to inhibit bacterial growth in H37Rv-infected MyD88^{-/-} macrophages (*Figure 2-5B*). These findings were unexpected because they suggested that the traditional markers of activation did not correctly predict iNKT cell antimycobacterial function.

We next addressed whether CD1d expression by the infected macrophages was required. iNKT cells were able to limit intracellular bacterial growth only if the infected macrophages expressed CD1d (*Figure 2-6A*). Normalizing the bacterial growth in each experiment allowed us to determine the requirement for CD1d across multiple experiments (see

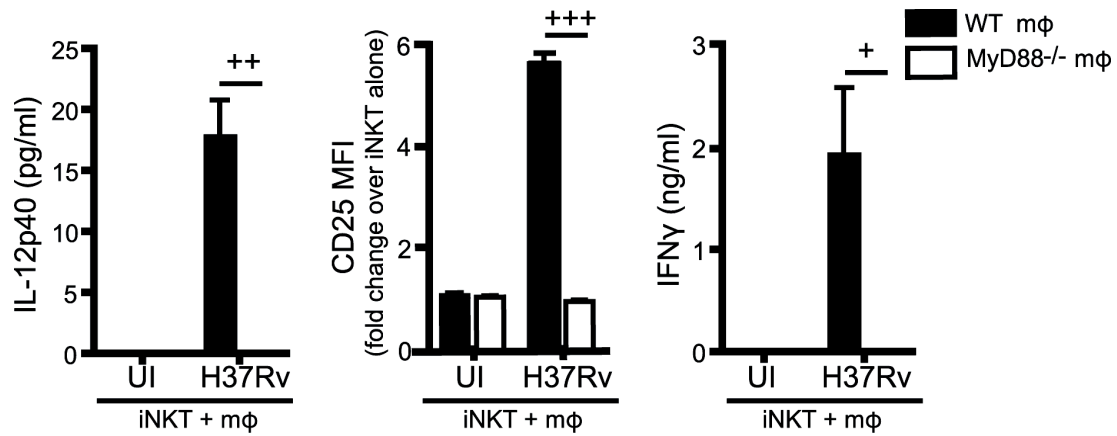


Figure 2-4. iNKT cell activation is decreased by MyD88^{-/-} Mtb-infected mφ.

iNKT cells were cultured with uninfected or H37Rv-infected WT or MyD88^{-/-} mφ for 24 hours. Cells were stained for CD25 and mφ were distinguished from iNKT cells by F4/80 staining. Supernatant was harvested at 24 hours and IFNγ and IL-12p40 measured by ELISA. Error bars indicate mean ± SEM. +P < 0.05, ++P < 0.01, +++P < 0.001 (unpaired Student's t-test). Data are representative of two independent experiments.

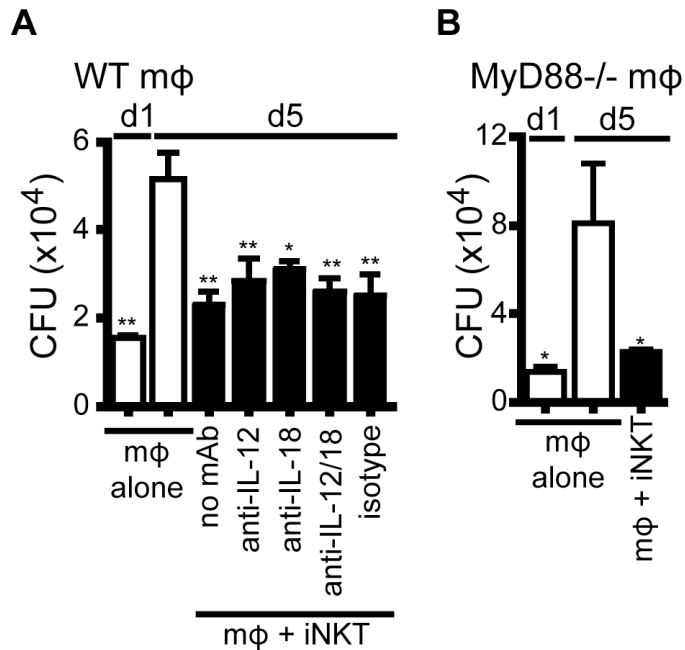


Figure 2-5. iNKT cell mediated control does not require IL-12 or IL-18.

(A) Colony forming unit (CFU) assay measuring Mtb bacterial growth in H37Rv-infected WT mφ on d1 and d5 post-infection. iNKT cells, anti-IL-12p40, anti-IL-18 blocking or isotype control antibodies were added on d1 after infection. (B) CFU assay d1 and d5 post-infection for H37Rv-infected MyD88^{-/-} mφ with iNKT cells added on d1. Error bars indicate mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 (One-way ANOVA with Dunnett's post-test, compared to d5 untreated mφ). Data are representative of two independent experiments with three or more replicates.

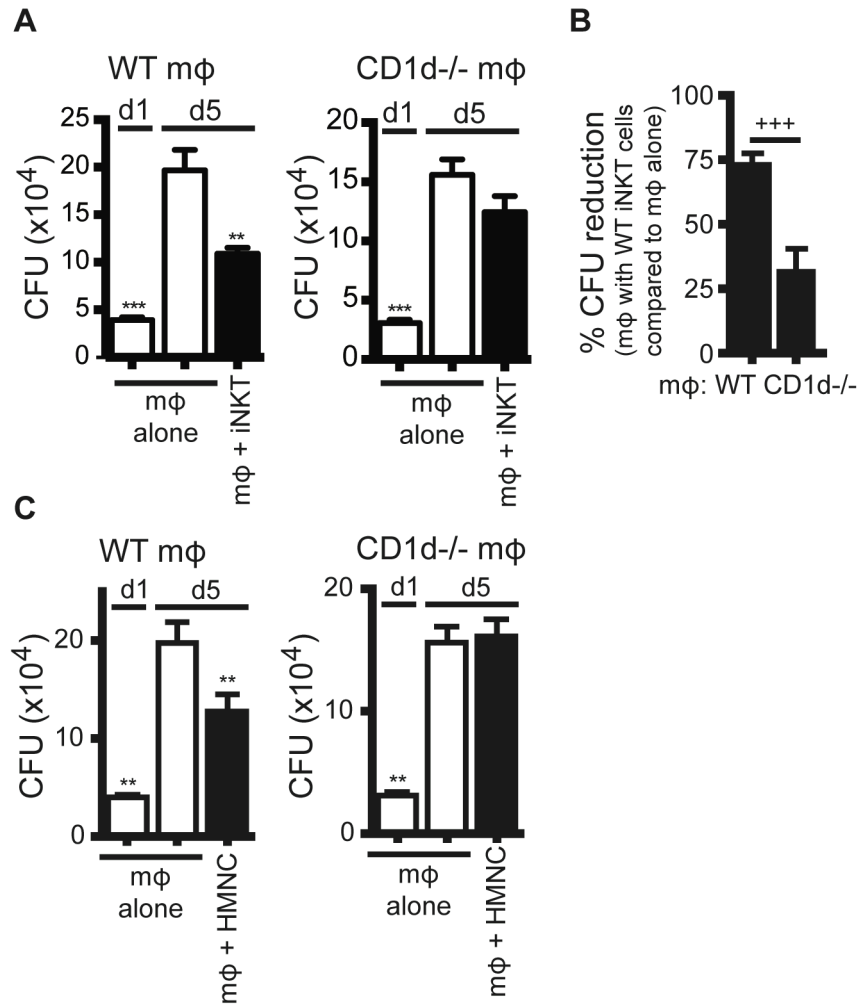


Figure 2-6. iNKT cell mediated control is CD1d-dependent.

(A, C) CFU assay d1 and d5 post-infection for H37Rv-infected WT and CD1d^{-/-} mφ with iNKT cells added on d1 at a ratio of 1:1 (A) or HMNC at a ratio of 3:1 (C). (B) Compiled data from 6 independent experiments as described in (A). Error bars indicate mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 (One-way ANOVA with Dunnett's post-test, compared to d5 untreated mφ). +++P < .001 (unpaired Student's t-test). Data are representative of six (A, B) and one (C) independent experiment(s) with three or more replicates.

Experimental Procedures for details). When Mtb growth inhibition by iNKT cells in WT and CD1d^{-/-} macrophages were compared in this manner, iNKT cells suppressed growth of Mtb in WT macrophages significantly more than in CD1d^{-/-} macrophages (73.1 ± 4.7% CFU reduction vs. 31.5 ± 9.3%) (mean ± SEM, n=6 experiments, p=0.0002) (*Figure 2-6B*). To confirm the dependence on CD1d for bacterial control by iNKT cells, and to show that the culture conditions of the primary cell lines had not significantly altered the effector functions of the iNKT cells, we repeated this experiment using HMNC as a source of primary uncultured iNKT cells. We found that HMNCs also required CD1d signaling for inhibition of bacterial growth (*Figure 2-6C*).

Since CD1d expression by macrophages was required to elicit optimal iNKT cell effector function, we considered whether Mtb infection altered the CD1d surface expression on macrophages. After 24 hours, H37Rv infection led to a modest increase in CD1d surface expression on macrophages (1.5 ± 0.1 fold change) (mean ± SEM, n=13 experiments, p<0.0001) (*Figure 2-7*). Co-culture with iNKT cells led to a slight increase in CD1d expression during H37Rv infection (1.9 ± 0.1 fold change) (mean ± SEM, n=22 experiments, p<0.0001) (*Figure 2-7*). These increases were averages for the bulk culture and may have underestimated the increase for individually infected macrophages, because not every macrophage was infected.

These results indicated that the cytokines (IL-12, IL-18) that drive expression of CD25 and IFN γ production were not required to stimulate iNKT cell antimycobacterial effector functions. In contrast, CD1d expression, which played only a small role in stimulating IFN γ production, was required for iNKT cell mediated control of intracellular Mtb growth. The dissociation between standard measurements of iNKT cell activation from iNKT cell effector function in response to Mtb infection was unexpected.

iNKT cells exhibit an antimycobacterial function independent of IFN γ

Since IL-12 and IL-18 blockade largely inhibited IFN γ production but had little impact on CFU control, we considered whether iNKT cell antimicrobial effector function was independent of IFN γ . To test this, we derived an IFN γ ^{-/-} iNKT cell line. When IFN γ ^{-/-} iNKT cells were added to

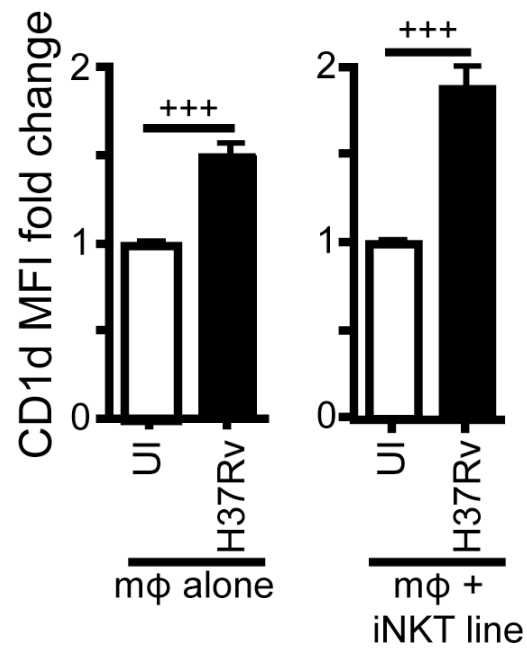


Figure 2-7. Mφ CD1d expression increases with Mtb infection.

H37Rv-infected mφ after 24 hours. CD1d MFI fold change over uninfected mφ either without or with iNKT cells. Error bars indicate mean \pm SEM. +++P < .001 (unpaired Student's t-test). Data are compiled from more than 12 independent experiments.

H37Rv-infected macrophages, they inhibited bacterial growth similar to WT iNKT cells (*Figure 2-8A*). Over multiple experiments, WT and IFN γ ^{-/-} iNKT cells reduced bacteria growth similarly by d5 by 60.0 ± 4.7% versus 60.1 ± 4.0%, respectively (mean ± SEM, n=13 experiments, p=NS) (*Figure 2-9*). A similar result was found by d7 post-infection (*Figure 2-9*).

IFN γ plays an important antimicrobial role during Mtb infection. To be certain that iNKT cells were controlling bacterial growth independently of IFN γ signaling, WT and IFN γ ^{-/-} iNKT cells were cultured with H37Rv-infected IFN γ R^{-/-} macrophages. The iNKT cells still limited bacterial growth by d5 in these cells (*Figure 2-8B*). We also examined whether iNKT cells controlled Mtb growth through nitric oxide, an important mediator of antimycobacterial immunity produced during infection by IFN γ stimulation of the enzyme inducible nitric oxide synthase (iNOS)²⁸. Neither IFN γ nor nitrite, a stable breakdown product of nitric oxide, were detected after addition of IFN γ ^{-/-} iNKT cells, while both were detected when WT iNKT cells were present (*Figure 2-8C*). Furthermore, naïve splenocytes (as a source of iNKT cells¹⁸) inhibited bacterial replication similarly in WT, IFN γ R^{-/-}, and iNOS^{-/-} macrophages (data not shown).

We next tested whether the IFN γ ^{-/-} iNKT cells also required CD1d to mediate their antimicrobial effector function. Similar to WT iNKT cells, IFN γ ^{-/-} iNKT cells inhibited growth of Mtb in WT macrophages significantly more than in CD1d^{-/-} macrophages by 73.3 ± 7.2% versus 34.4 ± 10.6% (mean ± SEM, n=5 experiments, p=0.0043) (*Figure 2-8D*). These data demonstrated that iNKT cells were capable of controlling Mtb growth independently of IFN γ and nitric oxide and that the IFN γ -independent effector function required CD1d-mediated activation.

iNKT cell antimicrobial function is independent of cytolytic activity and TNF

Human iNKT cells stimulated with α GalCer were shown to control Mtb infection in human monocyte-derived macrophages through granulysin-mediated cytolytic activity¹⁶. Based on this finding, we determined whether iNKT cells, in the absence of additional stimulation, inhibited Mtb growth through cytolytic activity. To block the major pathways of cytolysis, we added Perforin^{-/-} (Pfn^{-/-}) iNKT cells to H37Rv-infected WT macrophages (*Figure 2-10A*). In addition, we added

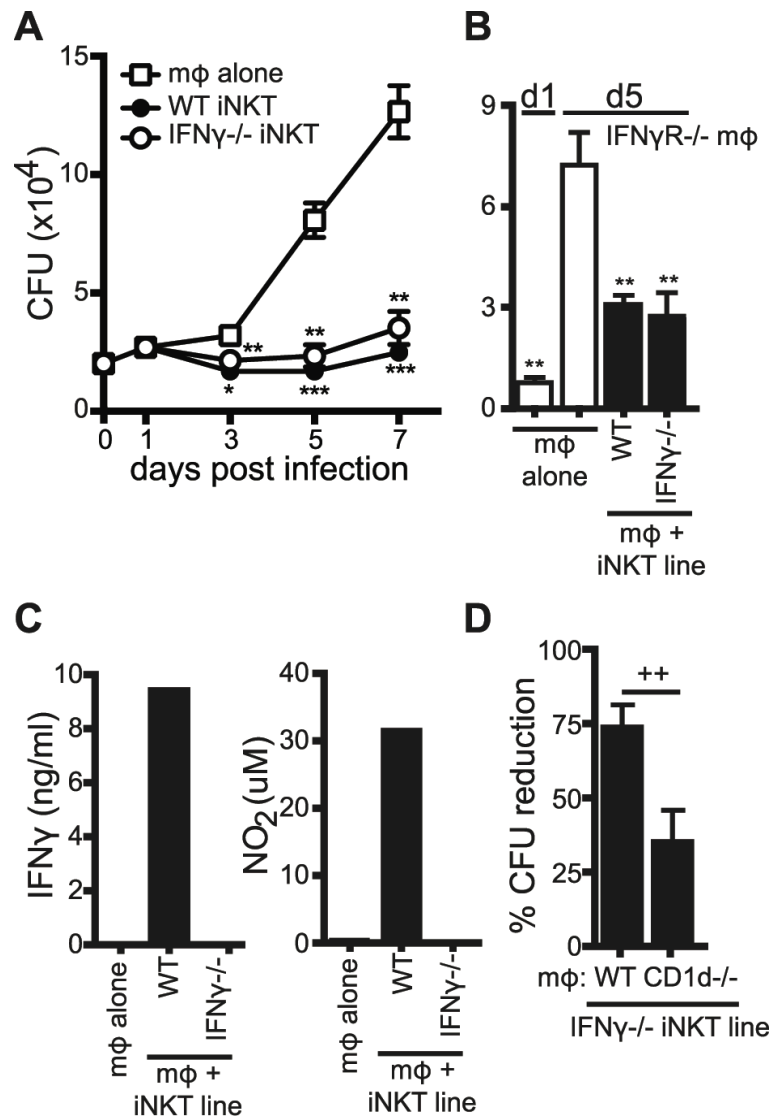


Figure 2-8. iNKT cell mediated control is independent of IFN γ .

(A) CFU for H37Rv-infected WT mφ with WT and IFN γ ^{-/-} iNKT cells added on d1 post-infection. CFU were measured on d0 (mφ alone) and on d1, d3, d5 and d7 post-infection. (B) CFU assay d1 and d5 post-infection for H37Rv-infected IFN γ R^{-/-} mφ with WT and IFN γ ^{-/-} iNKT cells added on d1. (C) IFN γ and nitrite, a stable breakdown product of NO₂, measured for H37Rv-infected WT mφ with WT and IFN γ ^{-/-} iNKT cells added on d1 post-infection. (D) Compiled data from 5 independent experiments of the CFU assay d1 and d5 post-infection with H37Rv-infected WT and CD1d^{-/-} mφ with IFN γ ^{-/-} iNKT cells added on d1. Error bars indicate mean \pm SEM. *P < 0.05, **P < 0.01, ***P < .001 (One-way ANOVA with Dunnett's post-test, compared to d3, d5, or d7 untreated mφ). ++P < .01 (Unpaired Student's t-test). Data are representative of or cumulative from 13 (d5) and four (d7) (A), two (B) three (C), or four (D) independent experiments with three or more replicates.

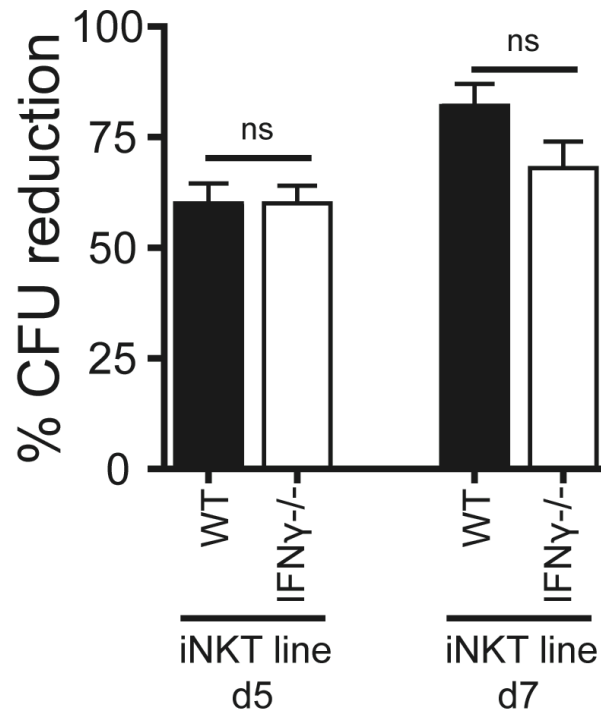


Figure 2-9. WT and IFN γ ^{-/-} iNKT cells inhibit Mtb growth at comparable levels.

Compiled data from CFU assays d5 and d7 post-infection for H37Rv-infected WT m ϕ with WT or IFN γ ^{-/-} iNKT cells added on d1. The CFU reduction for WT and IFN γ ^{-/-} iNKT cells on d5 was 60.0 \pm 4.7%, and 60.1 \pm 4.0%, respectively. On d7, the CFU reduction was 81.9 \pm 5.4%, and 67.9 \pm 6.2%, respectively. Error bars indicate mean \pm SEM. (Unpaired Student's t-test, p = NS). The data are compiled from 13 (d5) and four (d7) independent experiments.

either WT or IFN γ ^{-/-} iNKT cells to Fas^{-/-} macrophages (*Figure 2-10B*). Under these conditions, iNKT cells still significantly suppressed Mtb growth in macrophages. To eliminate the possibility of redundancy between cytolytic pathways, Pfn^{-/-} iNKT cells were added to H37Rv-infected Fas^{-/-} macrophages, and despite elimination of both of the dominant CTL pathways, iNKT cells were still able to significantly inhibit bacterial growth (data not shown). These data show that the major cytolytic pathways were not required for iNKT cell antimicrobial function in this system.

We also took an alternative approach to inhibit cytolytic activity by blocking an important downstream effector of cytolytic activity, caspase-3. Using a specific peptide inhibitor to inhibit caspase-3 activity and apoptosis in target cells, we found that addition of caspase-3 inhibitor to co-cultures of IFN γ ^{-/-} iNKT cells and H37Rv-infected macrophages, at concentrations that block apoptosis of infected macrophages²⁹, did not affect the ability of IFN γ ^{-/-} iNKT cells to inhibit bacterial growth (*Figure 2-10C*).

TNF plays an important antimicrobial role during Mtb infection³⁰ and we detected TNF, albeit at low levels, in our co-culture system (data not shown). Therefore, we used TNFR1/2^{-/-} macrophages, which lack both TNF receptors, to test whether TNF signaling was required for iNKT cell control of Mtb. Both WT and IFN γ ^{-/-} iNKT cells inhibited bacterial growth when cultured with H37Rv-infected TNFR1/2^{-/-} macrophages, indicating that TNF did not mediate the antimicrobial effector function of IFN γ ^{-/-} iNKT cells (*Figure 2-10D*). Finally, based on the newly appreciated role of IL-1 β in limiting growth of Mtb in macrophages^{31, 32}, we determined whether IL-1 β mediated iNKT cell control of bacterial growth. IFN γ ^{-/-} iNKT cells cultured with IL-1R^{-/-} macrophages controlled Mtb growth, ruling out a role for IL-1 β signaling (data not shown).

These data showed that iNKT cells controlled Mtb growth independently of cytolytic activity and cytokines previously identified as antimycobacterial, TNF and IL-1 β . They suggested that iNKT cells limit bacterial growth through a non-classical effector function.

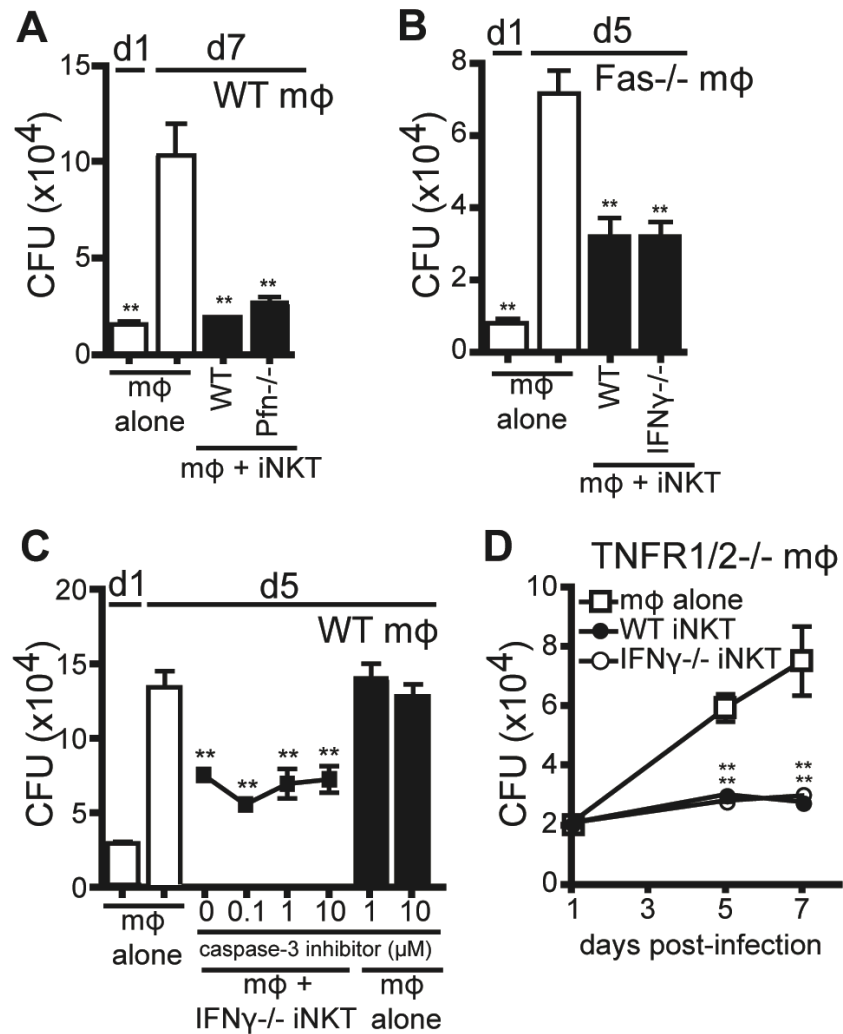


Figure 2-10. IFN γ -independent antimicrobial effector function of iNKT cells is independent of cytolytic activity.

(A-D) CFU assay d1, d5, and/or d7 post-infection with H37Rv-infected WT mφ (A, C), Fas^{-/-} mφ (B), or TNFR1/2^{-/-} mφ (D) with WT iNKT cells (A, B, D), IFN γ ^{-/-} iNKT cells (B-D), or Prf^{-/-} iNKT cells (A) added on d1 post infection at a 1:1 ratio. (C) H37Rv-infected mφ were treated with 0.1-10 μ M of caspase-3 inhibitor peptide (Z-DEVD-FMK) 2 hours prior to addition of iNKT cells. Error bars indicate mean \pm SEM. *P < 0.05, **P < 0.01 (One-way ANOVA with Dunnett's post-test, compared to d5 or d7 untreated mφ.) Data are representative of three (A, C, D) or two (B) independent experiments with three or more replicates.

iNKT cells secrete a soluble factor with antimicrobial properties

We next determined whether the antimicrobial activity was a soluble factor or cell contact dependent. Using a transwell system, we found that both WT and IFN γ ^{-/-} iNKT cells placed in trans from H37Rv-infected macrophages were able to suppress Mtb growth (*Figure 2-11*). Importantly, this effect was only observed if the iNKT cells were in contact with other macrophages in the transwell, but not when the iNKT cells were cultured alone. This was consistent with our observation that induction of the iNKT cell effector function required CD1d signaling via macrophage contact. The finding that contact between iNKT cells and uninfected macrophages was sufficient to induce the antimicrobial activity raised the possibility that a mediator secreted by infected macrophages (e.g., in trans) augmented iNKT cell activation, either by increasing macrophage CD1d expression or by costimulating iNKT cell activation. Most importantly, this experiment confirmed that iNKT cells required cell contact and CD1d expression for their activation, and indicated that their antimicrobial activity was mediated by a soluble factor.

We also found that α GalCer stimulation of IFN γ ^{-/-} iNKT cells cultured in trans boosted their ability to control bacterial growth (data not shown). Furthermore, conditioned media produced from IFN γ ^{-/-} iNKT cells stimulated with α GalCer-loaded WT but not CD1d^{-/-} macrophages stimulated bacterial control when added to macrophages (*Figure 2-12B*). We then used conditioned media samples derived from IFN γ ^{-/-} iNKT cells stimulated by unloaded or α GalCer-loaded WT or CD1d^{-/-} macrophages for screening purposes. These samples were size fractionated at 10kDa and 50kDa MW cutoffs (*Figure 2-12A*). We identified several cytokines present at high levels only in the fractions that had antimicrobial activity: GM-CSF, TNF, and IL-4 (*Figure 2-12C*). Since TNF had been previously eliminated and IL-4 is not known to enhance bacterial control³³, we investigated whether iNKT cells produced GM-CSF after co-culture with Mtb-infected macrophages and whether it had antimycobacterial activity.

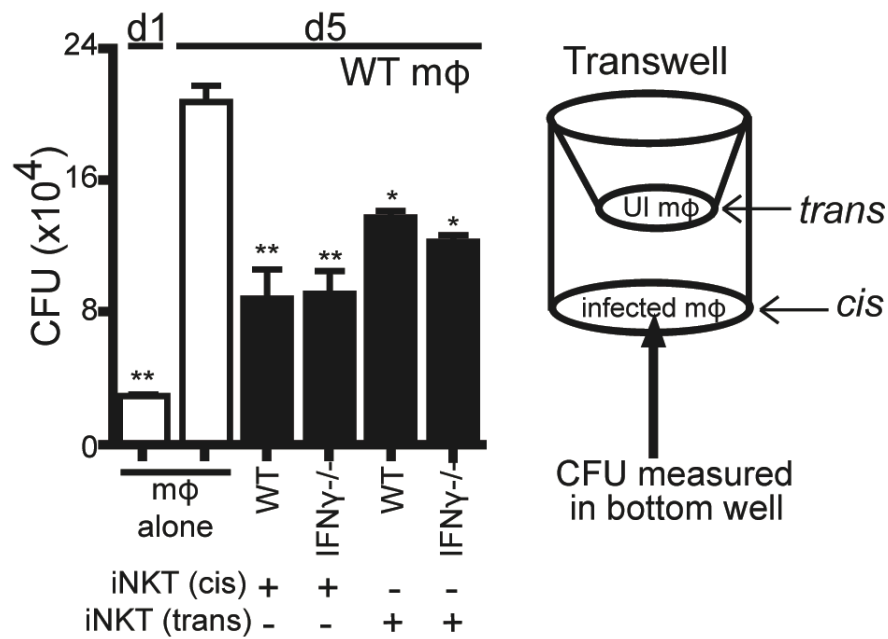


Figure 2-11. The antimicrobial effector function of iNKT cells is a soluble factor.

Transwell CFU assay for H37Rv-infected WT mφ in a 24-well plate with either WT or IFN $\gamma^{-/-}$ iNKT cells added directly (*cis*) or 0.4 μ m transwell inserts with WT or IFN $\gamma^{-/-}$ iNKT cells in the presence of uninfected WT mφ (*trans*) added on d1. Error bars indicate mean \pm SEM. *P < 0.05, **P < 0.01 (One-way ANOVA with Dunnett's post-test, compared to d5 untreated mφ.) Data are representative of two independent experiments with four replicates each.

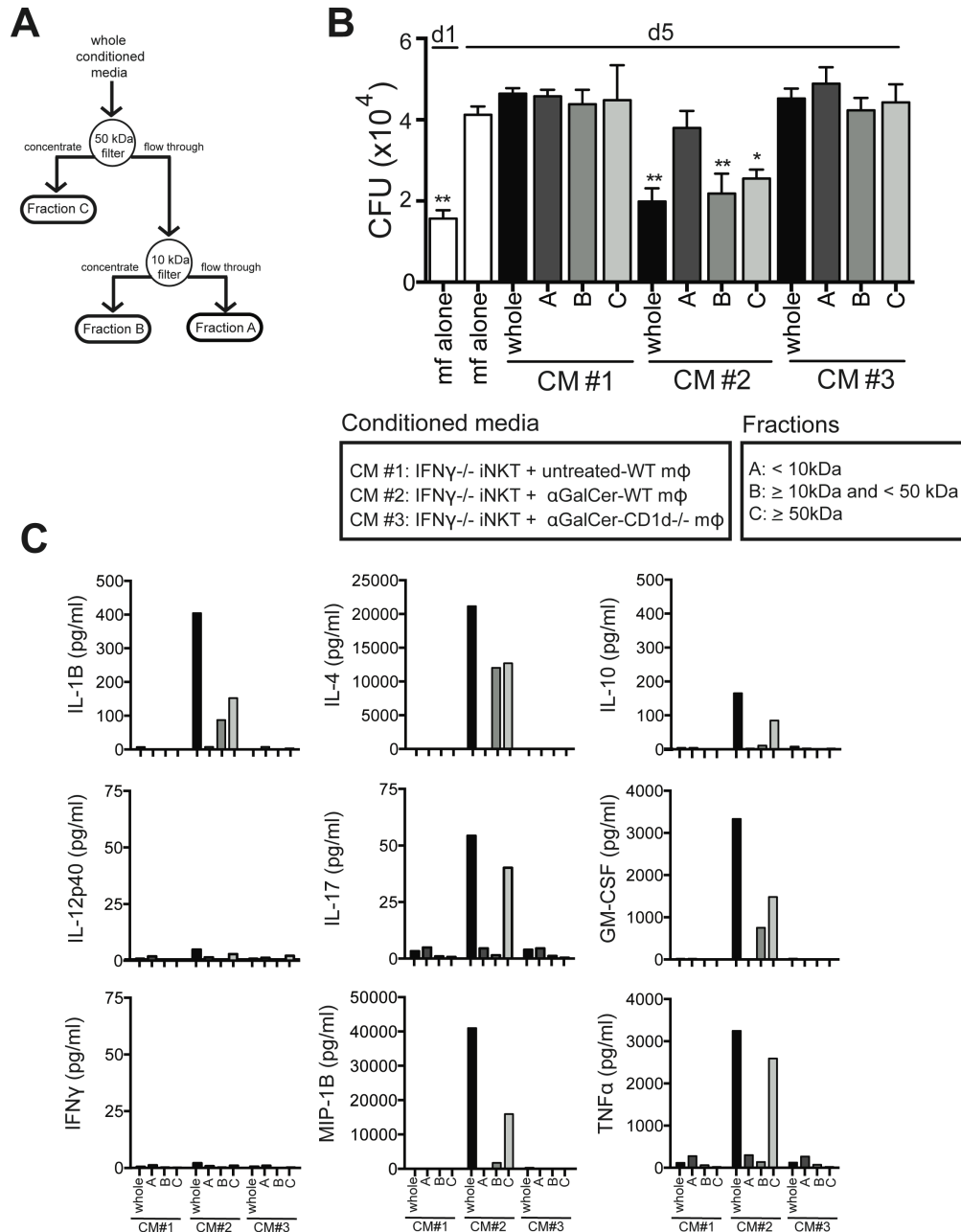


Figure 2-12. Conditioned media fractions from α -GalCer-stimulated IFN γ ^{-/-} iNKT cells inhibit bacterial growth.

(A) Size fractionation strategy for conditioned media samples using 50kDa and 10kDa Amicon Ultra-15 Centrifugal Filter Units. (B) CFU assay for H37Rv-infected WT mφ treated on d1 with whole and size fractionated conditioned media samples from IFN γ ^{-/-} iNKT cells stimulated for 24 hours with untreated or α GalCer-loaded WT or CD1d^{-/-} mφ at 1:50 dilution. (C) Cytokines measured in whole and size fractionated conditioned media samples from IFN γ ^{-/-} iNKT cells stimulated for 24 hours with untreated or α GalCer-loaded WT or CD1d^{-/-} mφ. Cytokines were measured using Bioplex immunoassay. Error bars indicate mean \pm SEM. *P < 0.05, **P < 0.01 (One-way ANOVA with Dunnett's post-test, compared to d5 untreated mφ). The data are representative of two independent experiments.

iNKT cells produce GM-CSF during Mtb infection in a CD1d-dependent manner and it is critical for controlling Mtb growth

We detected GM-CSF production 24 hours after co-culture of both WT and IFN γ ^{-/-} iNKT cells with H37Rv-infected macrophages (*Figure 2-13A, Figure 2-14A*). Because we had already established that the IFN γ -independent antimicrobial function was CD1d-dependent, we tested whether GM-CSF production by iNKT cells required CD1d expression. We found that IFN γ ^{-/-} iNKT cells produced significantly more GM-CSF when cultured with Mtb-infected WT macrophages than with Mtb-infected CD1d^{-/-} macrophages; however, some GM-CSF was produced even in the absence of CD1d, especially at higher Mtb MOI (*Figure 2-13B*).

To determine if GM-CSF was required for iNKT cell-mediated control in vitro, we added anti-GM-CSF blocking antibodies to the co-culture of IFN γ ^{-/-} iNKT cells and Mtb-infected macrophages. GM-CSF blockade impaired bacterial control by IFN γ ^{-/-} iNKT cells leading to a significant increase in CFU compared to both an isotype control and untreated conditions (*Figure 2-13C*). This suggested that GM-CSF was required for iNKT cell mediated control of Mtb infection in this model. When we added anti-GM-CSF blocking antibodies to co-cultures of WT iNKT cells and Mtb-infected macrophages, we observed a modest decrease in bacterial inhibition by WT iNKT cells in the GM-CSF blockade condition compared to untreated or isotype control, although it was not significant (*Figure 2-14B*). These results suggest that either the antimicrobial functions of GM-CSF are less crucial in the presence of IFN γ or that there is redundancy in the antimicrobial mechanisms of these two cytokines.

To determine if this iNKT cell effector pathway was also relevant for human cells, we tested the human iNKT cell clone J3N.5. The iNKT cells produced significantly more GM-CSF after co-culture with H37Rv-infected U937 cells, a human monocytic line, than with uninfected U937 cells and this was inhibited by addition of an anti-CD1d blocking antibody (*Figure 2-15*). J3N.5 also produced IFN γ in response to H37Rv-infected U937 cells, and this was similarly inhibited by an anti-CD1d blocking antibody (data not shown). Next, we determined whether iNKT cells produced GM-CSF during aerosol infection in vivo. We isolated cells from the lungs of mice

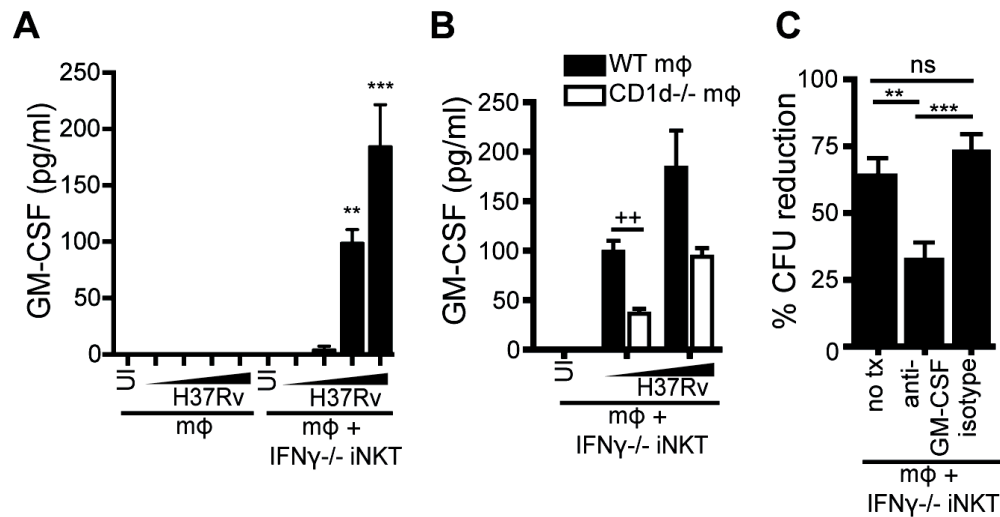


Figure 2-13. iNKT cells produce GM-CSF during Mtb infection in a CD1d-dependent manner and it is critical for controlling Mtb growth.

(A, B) IFNγ^{-/-} iNKT cells added to uninfected or H37Rv-infected WT (A, B) and CD1d^{-/-} mφ (B). Murine GM-CSF measured in supernatant harvested after 24 hours. (C) % CFU reduction calculated from CFU assays for H37Rv-infected WT mφ with IFNγ^{-/-} iNKT cells added on d1 in the presence of anti-GM-CSF blocking antibody (10-50 μg/ml) or isotype control. Error bars indicate mean ± SEM. ++P < .01 (unpaired Student's t-test, WT versus CD1d^{-/-} macrophages (B)). **P < 0.01, ***p < 0.001 (One-way ANOVA with Tukey post-test (C), and Dunnett's post-test (A), compared to uninfected mφ). Data are representative of or compiled from four (A, C) and two (B) independent experiments with three or more replicates each ▲, MOI titration approximately 0.5:1 - 3:1 (A, B).

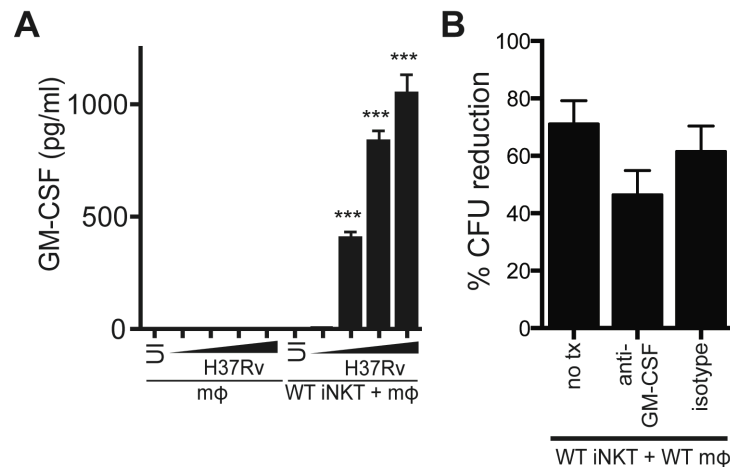


Figure 2-14. WT iNKT cells produce GM-CSF following Mtb infection and control is limited by anti-GM-CSF blocking antibody.

(A) Murine GM-CSF measured in supernatants after 24-hr co-culture of WT iNKT cell line with uninfected and H37Rv-infected WT mφ. (B) WT iNKT cells were added to H37Rv-infected WT mφ on d1 without additives, or in the presence of anti-GM-CSF blocking antibody or nonspecific isotype control antibody (25 μg/ml). CFU were determined on d5. Error bars indicate mean ± SEM. ***P < 0.001 (One-way ANOVA with Dunnett's post-test, compared to uninfected mφ). The data are representative (A) or compiled from (B) three independent experiments. Statistical analysis was performed using a one-way ANOVA and was not significant.

infected with virulent Mtb at serial time points after infection. Pulmonary iNKT cells were identified by TCR β and CD1d-tetramer staining and their production of GM-CSF and IFN γ was assessed by intracellular cytokine staining (ICS) directly ex vivo without further stimulation. The frequency of iNKT cells in the lung did not change greatly over the course of infection, although the total number of iNKT cells increased in parallel with the overall increase in T cell recruitment to the lung (*Figure 2-16A and 2-16B*). Similar to our in vitro observations, CD69 expression increased on iNKT cells in the lung over the course of infection (*Figure 2-16C*). A small percentage of iNKT cells in the lungs of naive mice secreted GM-CSF. By two weeks post-infection, there was an increase in both the percentage and absolute number of iNKT cells producing GM-CSF; this was not the case for IFN γ (*Figure 2-17A and 2-17B*). At all time points examined post-infection, a significantly greater percentage of iNKT cells in the lung produced GM-CSF than IFN γ . For example, at week 2, $11.0 \pm 1.3\%$ iNKT cells were GM-CSF⁺ compared to $1.0 \pm 0.3\%$ IFN γ ⁺ iNKT cells (mean \pm SEM, $p < 0.0001$). These data demonstrated that iNKT cells are an early source of GM-CSF in the lung during Mtb infection and underscored our in vitro observation that GM-CSF and IFN γ production by iNKT cells are regulated differently during Mtb infection.

In order to evaluate the role of CD1d signaling in the production of GM-CSF by iNKT cells in vivo, we used an adoptive transfer model, in which fluorescently-labeled iNKT cells were injected iv into Mtb-infected WT or CD1d^{-/-} recipients. Downregulation of the iNKT cell TCR usually limits the ability to detect iNKT cells in vivo. This transfer model allowed monitoring of iNKT cells in vivo without the need for CD1d tetramer staining. We detected significantly less GM-CSF and IFN γ production by iNKT cells transferred into Mtb-infected CD1d^{-/-} hosts compared to WT hosts, while CD69 expression was induced on iNKT cells transferred into both WT and CD1d^{-/-} recipients (*Figure 2-17C*). Interestingly, we observed a strong CD1d-dependent IFN γ response by the transferred iNKT cells, which we did not detect in the intact mice (*Figure 2-17A and 2-17B*). The transferred iNKT cells were likely to have a stronger response and a lower threshold for activation due to their prior stimulation in vitro. There is recent data, discussed below, that

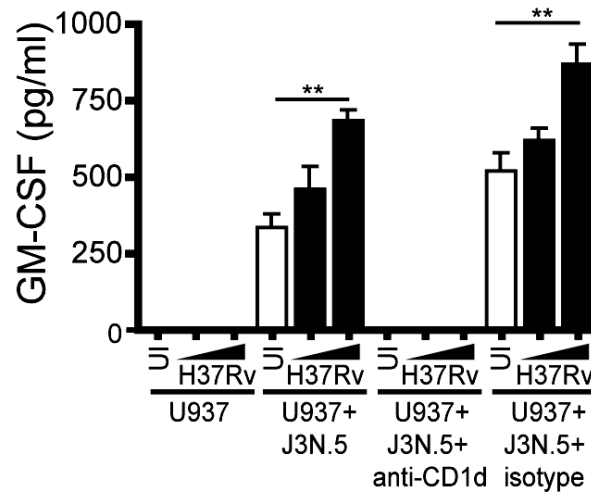


Figure 2-15. Human iNKT cell clones produce GM-CSF in response to Mtb infection.

J3N.5 human iNKT cell clone added to uninfected or H37Rv-infected U937 cells in the presence of anti-human-CD1d blocking antibody or isotype control. Human GM-CSF measured in supernatant harvested after 24 hours. Error bars indicate mean \pm SEM. ** $P < 0.01$ (One-way ANOVA with Dunnett's post-test (D) compared to uninfected m ϕ). Data are representative of three independent experiments with three or more replicates each ▲, MOI titration approximately 2:1 – 10:1.

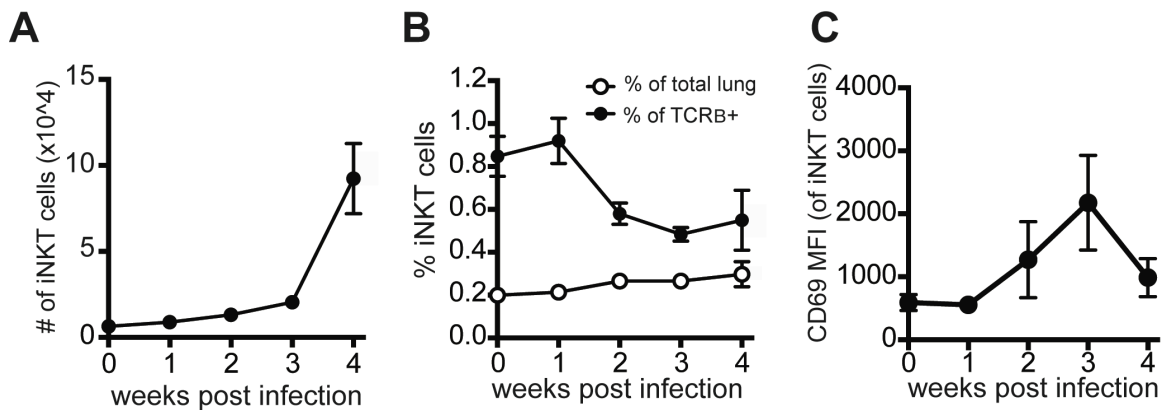


Figure 2-16. iNKT cells are found in the lung after aerosol Mtb infection.

Lung mononuclear cells from WT Mtb-infected mice were stained and fixed. iNKT cells were identified as TCRB $^+$ and CD1d-tetramer $^+$. Number (A) and percentage (B) of iNKT cells in the lung were measured. (C) CD69 MFI measured on iNKT cell subset. The data are representative of two independent experiments with 5 mice each.

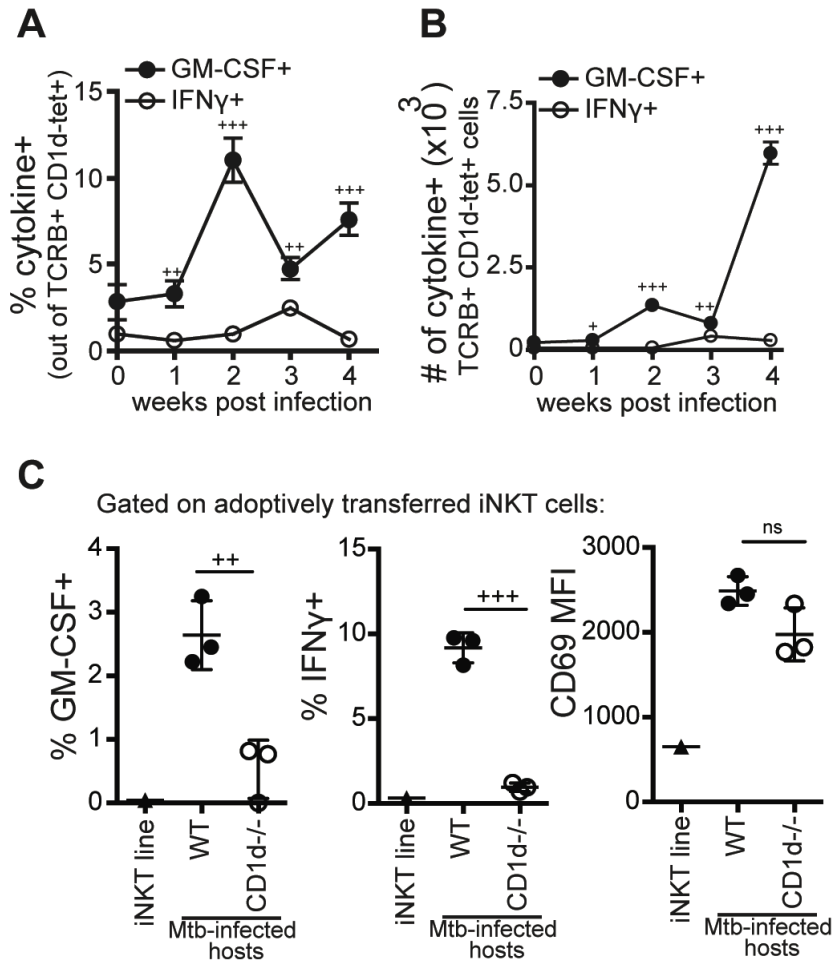


Figure 2-17. iNKT cells produce GM-CSF in the lungs of Mtb-infected mice in a CD1d-dependent manner

(A, B) Lung mononuclear cells from WT Mtb-infected mice were incubated with brefeldin A for four hours at 37°C and then stained. iNKT cells were identified as TCR β ⁺ and CD1d-tetramer⁺. Percentage (A) and number (B) of iNKT cells producing GM-CSF and IFN γ . (C) % GM-CSF⁺, % IFN γ ⁺, and CD69 MFI for iNKT cells transferred iv into WT or CD1d^{-/-} Mtb-infected hosts and iNKT cells cultured for 20 hours in basic media. Lung mononuclear cells were treated and stained as in (A, B). Transferred iNKT cells were distinguished from host cells by eFluor 450 staining. Error bars indicate mean \pm SEM. +P < .05, ++P < .01, +++P < .001 (unpaired Student's t-test, GM-CSF⁺ versus IFN γ ⁺ for respective time points (A, B), WT versus CD1d^{-/-} hosts (C)). Data are representative of two independent experiments with 5 mice each (A, B) or three independent experiments with three or more replicates each (C).

epigenetic modifications at the IFNG locus of iNKT cells may explain why IFN γ is only detected after priming by a strong stimulus³⁴. The observation that the in vivo IFN γ response was almost entirely CD1d-dependent suggests that our in vitro infection model may be over-estimating the effect of cytokine-driven stimulation. This experiment also confirmed our in vitro results that activation marker expression and effector functions could be elicited by distinct activating pathways in iNKT cells.

These data showed that both murine and human iNKT cells produced GM-CSF upon recognition of Mtb infection in a CD1d-dependent manner and GM-CSF was a critical component of iNKT cell antimycobacterial function in vitro.

GM-CSF is sufficient to inhibit Mtb growth

We next determined whether GM-CSF alone was sufficient to mediate control of Mtb growth. In a dose-dependent manner, recombinant GM-CSF added to infected macrophages was sufficient to inhibit Mtb growth (*Figure 2-18*). Recombinant GM-CSF has also previously been reported to inhibit the growth of mycobacterium in human monocyte-derived macrophages (MDM)^{35, 36, 37}. The data showed that GM-CSF was sufficient to inhibit bacterial growth in murine macrophages.

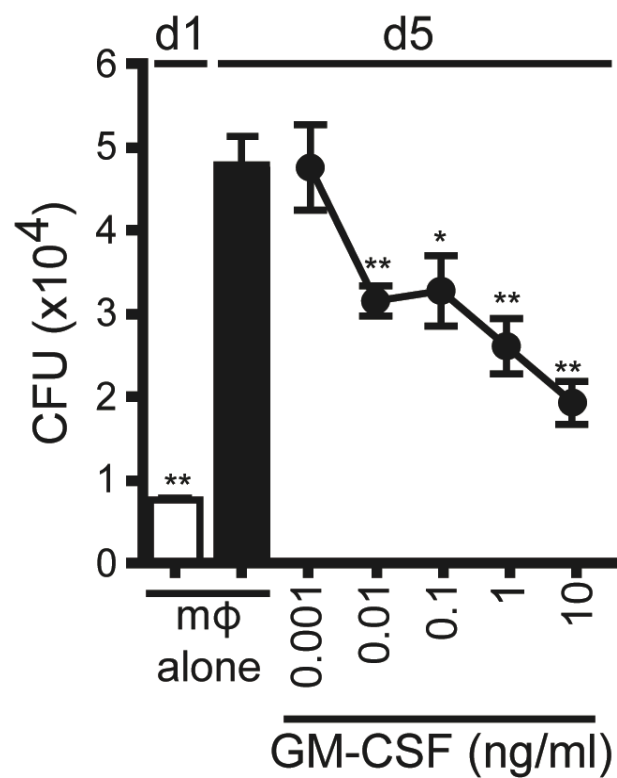


Figure 2-18. GM-CSF is sufficient to inhibit Mtb growth in mφ.

CFU assay for H37Rv-infected murine WT mφ treated with recombinant GM-CSF from 0.001-10 ng/ml concentration on d1. Error bars indicate mean \pm SEM. *P < .05, **P < 0.01 (One way ANOVA with Dunnett's post-test, compared to d5 untreated mφ). Data are representative of four independent experiments.

DISCUSSION

We find that iNKT cells cultured with Mtb-infected macrophages inhibit intracellular bacterial growth¹⁸. This is the only model of which we are aware that allows investigation of the direct antimicrobial effector function of iNKT cells. In the presence of Mtb-infected macrophages, iNKT cells became activated and upregulated the activation markers, CD69 and CD25, and produced IFN γ . While IL-12 and IL-18 produced by infected macrophages induced CD25 and IFN γ expression by iNKT cells, these cytokine signals were not required for iNKT cell control of Mtb. In addition, macrophage expression of CD1d was dispensable for the upregulation of CD25 and only a minor factor in promoting IFN γ production, yet was essential to elicit iNKT cell antimycobacterial activity.

The ability to dissociate iNKT cell activation and IFN γ production from iNKT cell antimicrobial function uncovered a novel antibacterial function of iNKT cells: GM-CSF production. We showed that CD1d-mediated activation was crucial for the production of GM-CSF in response to Mtb infection by both murine and human iNKT cells in vitro, and by murine iNKT cells in vivo. In the absence of IFN γ , GM-CSF was essential for iNKT cell mediated inhibition of Mtb growth, and GM-CSF alone was sufficient for bacterial control in vitro. Interestingly, under conditions where either CD1d signaling or GM-CSF was blocked during iNKT cell co-culture there was still on average a 30% reduction in CFU. In the case of antibody blocking, inefficient inhibition during the five day assay may be a technical confounder. In addition, these data point to two alternative biological explanations. First, signals apart from TCR activation may contribute to the production of GM-CSF by iNKT cells (*Figure 2-13B*). Cytokines such as IL-12 plus IL-18 stimulate iNKT cells to produce GM-CSF (data not shown). Second, there may be molecules other than GM-CSF and IFN γ that are produced by iNKT cells and activate infected macrophages to inhibit bacterial growth. While GM-CSF is unlikely to be the only iNKT cell effector function that inhibits Mtb replication, our experiments demonstrate that it is a dominant antimicrobial pathway during Mtb infection.

Although signaling by IFN γ is crucial for control of Mtb, clinical data show that IFN γ present at the site of ongoing infection is inadequate to clear bacteria and IFN γ levels produced by CD4 $^{+}$ T cells do not correlate with disease progression or protection provided by BCG vaccination^{38, 39, 40}. An implication of these studies is that alternative pathways exist that control Mtb. A number of studies have demonstrated IFN γ -independent mechanisms of control by CD4 $^{+}$ and CD8 $^{+}$ T cells^{41, 42, 43}. Using iNKT cells as a model, we evaluated IFN γ -independent pathways of control in the innate immune compartment.

Two previous studies find GM-CSF to be required for host resistance in vivo based on the greater susceptibility of GM-CSF $^{-/-}$ mice compared to WT mice^{44, 45}. Although alveolar macrophages and type II epithelial cells were presumed to be the dominant source of GM-CSF in the lung, ectopic expression of GM-CSF driven by the surfactant C promoter did not fully rescue the susceptibility of GM-CSF $^{-/-}$ mice, suggesting that GM-CSF from other sources might be important for immunity. T cells are an important source of GM-CSF, and in addition to iNKT cells, other innate-like T cells produce GM-CSF during Mtb infection (*Chapter 3*). We have also found that conventional CD4 $^{+}$ T cells in the lungs of Mtb infected mice produce GM-CSF and may replace innate lymphocytes as a source of GM-CSF as the immune response to Mtb evolves. This may be one explanation for why iNKT cells are redundant during Mtb infection. Although GM-CSF restricts bacterial replication in human macrophages, its role as an effector molecule during clinical infection is harder to discern. Importantly, recent clinical data indicates that the development of anti-GM-CSF neutralizing antibodies are a form of acquired immunodeficiency associated with cryptococcal meningitis and pulmonary tuberculosis in otherwise normal individuals⁴⁶. This suggests that inhibition of GM-CSF signaling may increase clinical susceptibility to Mtb and other pulmonary pathogens.

To put our in vitro experiments in context, cytokines induced by Mtb-infected macrophages, which include IL-12 and IL-18, are drivers of IFN γ and other cytokine production by iNKT cells, independent of TCR activation (e.g., in an antigen-independent manner). An important question is whether this mechanism is relevant in vivo. In vitro, our assays use numerous

macrophages that are uniformly infected, with the consequence that the effective cytokine concentration that the iNKT cells are exposed to could be higher than is relevant in vivo. Furthermore, the iNKT cell lines are primed to produce IFN γ because they have been repeatedly stimulated in vitro with α GalCer (see below). Interestingly, the iNKT cell lines produced both IFN γ and GM-CSF after short-term adoptive transfer into Mtb-infected mice, but only in a CD1d-dependent manner. Cytokine production was only observed when the iNKT cells were transferred into Mtb-infected WT mice but not when transferred into infected CD1d^{-/-} mice. Thus, although CD1d-independent GM-CSF and IFN γ was observed in vitro, TCR signaling is crucial in vivo during infection to stimulate iNKT cells to produce cytokines.

After a strong stimulus such as α GalCer, human iNKT cell clones produce both GM-CSF and IFN γ ; in contrast, GM-CSF dominates after a weak or autoreactive stimulus⁴⁷. GM-CSF production by iNKT cells plays an important role in the maturation of DC, which has been linked to effective T cell priming^{47, 48}. Wang et al found that in resting human iNKT cells the *CSF2* (GM-CSF) locus already had high histone H4 acetylation, indicative of chromatin availability, while the *IFNG* (IFN γ) locus had low histone H4 acetylation, which was increased only after strong stimulation³⁴. In contrast to the iNKT cell lines, we observed that endogenous polyclonal iNKT cells in the lungs of Mtb-infected mice more frequently produced GM-CSF than IFN γ , consistent with the idea that iNKT cells are poised to produce GM-CSF more readily than IFN γ , particularly when exposed to weak TCR agonists. This study further confirms that different iNKT cell effector functions may require different stimuli and that epigenetic modifications may explain at least part of this phenomenon.

It is clear that there is great heterogeneity in iNKT cell responses and that part of this variability comes from the diversity in activating stimuli iNKT cells encounter. We observed two distinct modes of activation that initiated different effector functions: (1) the IL-12/IL-18 pathway activated iNKT cells to produce IFN γ , and (2) a TCR-dependent pathway elicited antimycobacterial activity. Interestingly, in the context of Mtb infection, the conventional markers of iNKT cell activation did not correlate with effector function and our data indicate that GM-CSF

production may be a better marker of TCR-dependent iNKT cell effector function than IFN γ . Using transcriptional profiling, Cohen et al elegantly showed that iNKT cells share features of both NK cells and T cells⁴⁹. Here we show that during the host response to infection, different stimuli may trigger different iNKT cell effector programs characteristic of NK cells (e.g. activation by IL-12 and IL-18) or T cells (e.g. TCR activation). Future insight into iNKT cell immunity will require better understanding of how different activation stimuli can dictate subsequent effector functions and may facilitate the discovery of other novel antimicrobial roles for iNKT cells.

MATERIALS AND METHODS

Ethics Statement

All mice were bred and maintained using standard humane animal husbandry protocols. All animal experiments were performed in accordance with relevant guidelines for the care and handling of laboratory animals and were approved by the Dana Farber Cancer Institute Animal Care and Use Committee (Animal Welfare Assurance number A3023-01) under Public Health Service assurance of the Office of Laboratory Animal Welfare guidelines. Human blood collected from healthy donors was purchased from Research Blood Components (Boston, MA), and its use was approved by the Institutional Review Board of Brigham and Women's Hospital (Human Subjects Assurance FWA00000484). Written informed consent was obtained for each donor by Research Blood Components.

Mice

C57Bl/6 WT, IFN γ ^{-/-}, IFN γ R^{-/-}, TNFR1/2^{-/-}, Perforin^{-/-}, and Fas^{-/-} mice were obtained from Jackson Laboratories. Va14-Ja281 transgenic mice were provided by Dr. Albert Bendelac⁵⁰. CD1d^{-/-} mice were provided by Dr. Mark Exley⁵¹. MyD88^{-/-} mice were provided by Dr. Koichi Kobayashi⁵².

Macrophage isolation and culture

Thioglycollate (TGL)-elicited peritoneal macrophages were lavaged 4–5 days after 3% intraperitoneal TGL injection and then isolated by positive selection with CD11b microbeads and magnetic columns (Miltenyi Biotec). Purified cells were over 95% F4/80⁺ CD11b⁺, as determined by flow cytometry. Bone marrow-derived macrophages (BMDM) were differentiated from bone marrow after 7 days in RPMI supplemented with 20% L929 cell supernatant. U937 cell line was grown in complete media. Macrophages were seeded at 5x10⁵ cells/well in 24-well culture plates or 1x10⁵ in 96-well culture plates in complete RPMI 1640 medium (Invitrogen Life Technologies)

supplemented with 10% fetal calf serum (HyClone). For transwell assays, macrophages were seeded at 2×10^5 in 0.4 μ m cell culture inserts for 24-well plates (BD Bioscience).

iNKT cells and HMNC

iNKT cell lines were derived as previously reported⁵³. T cells were selected from splenocytes using the Pan T Isolation Kit (Miltenyi Biotec) and then cultured overnight at 37°C. The next day, T cells were labeled with PE-conjugated CD1d tetramer loaded with PBS-57 lipid antigen (National Institutes of Health Tetramer Core Facility) and sorted using anti-PE beads (Miltenyi Biotec). The purity of iNKT cells was higher than 95%. iNKT cells were then cultured with irradiated α GalCer-pulsed BMDCs in 24-well plates in complete RPMI medium with 10% FBS. Three to four days later, 1 ng/ml IL-2 (R&D Systems) and 10 ng/ml IL-7 (PeproTech) were added. iNKT cells were rested for at least 18 days before use. Human iNKT cell clones were derived and cultured as previously described⁷. Hepatic mononuclear cells (HMNCs) were isolated from mouse liver perfused with PBS and homogenized through 70 μ m cell strainer to single cell suspension. After centrifugation, the cells were resuspended in 30% Percoll and overlayed onto 80% Percoll layer (Sigma). The interface containing the lymphocytes was collected and washed before use.

Mtb in vitro culture and infection

H37Rv was grown and prepared as previously described¹⁸. Bacteria was counted and added to macrophages at an effective multiplicity of infection (MOI) of 0.2 for CFU experiments (or higher for ELISA and FACS assays) for two hours. Cultures were washed three times to remove extracellular bacteria. Infected macrophages were cultured overnight and iNKT cells or other conditions were added on d1. For CFU measurement, cells were lysed with 1% Triton X-100/PBS and lysate from quadruplicate conditions were plated in serial dilutions on Middlebrook 7H10 agar plates (ThermoFisher Scientific), and cultured at 37°C for 21 days. Infected macrophages were treated with the following reagents: caspase-3 inhibitor and caspase inhibitor negative control

(Calbiochem), anti-mouse-IL-12p40 (C17.8; Biolegend), anti-mouse-IL-18 (93-10C; MBL), anti-mouse-CD1d (19G112.2)⁵⁴, anti-human-CD1d (CD1d42; BD Pharmingen), anti-mouse-GM-CSF (MP1-22E9; Biolegend), recombinant murine GM-CSF (Peprotech), and IFN γ (murine, Peprotech; human, Biolegend). α GalCer was kindly provided by Gurdyal S. Besra.

% CFU reduction

To compare inhibition of bacterial growth across multiple experiments, % CFU reduction was calculated. 100% CFU reduction on d5 indicates complete inhibition of bacterial growth to d1 levels while 0% CFU reduction indicates no change in bacterial growth from untreated macrophages. $\% \text{ CFU reduction} = 100 \times [CFU_{(\text{untreated mf-d5})} - CFU_{(\text{treated mf-d5})}] / [(CFU_{(\text{untreated mf-d5})} - CFU_{(\text{untreated mf-d1})})]$

In vivo aerosol infections

In vivo infections were performed using virulent Mtb (Erdman strain). Mice infected with Mtb were housed under BSL3 conditions. For each infection, a bacterial aliquot was thawed, sonicated twice for 10 s, and then diluted in 0.9% NaCl/0.02% Tween 80. A 15-ml suspension of *M. tuberculosis* was loaded into a nebulizer (MiniHEART nebulizer; Vortran Medical Technology) and mice were infected via the aerosol route with a nose-only exposure unit (Intox Products) and received ~50–100 CFU/mouse. Mice were euthanized by CO₂ inhalation and lungs were aseptically removed after perfusion of 10 ml of sterile RPMI into the right ventricle of the heart. Lung mononuclear cells were obtained by mechanical disruption using a gentleMACS dissociator (Miltenyi Biotec) followed by incubation in collagenase (Sigma-Aldrich) for 30 mins at 37°C. Cells were isolated by forcing suspensions through a 70 μ M cell strainer and then enumerated in 4% trypan blue with a hemacytometer. Samples used for ICS were incubated for 4 hours at 37°C with IL-2 and Brefeldin A (GolgiPlug, BD Biosciences).

Adoptive transfer of iNKT cell lines

3-5x10⁶ iNKT cells were stained with Cell Proliferation Dye eFluor® 450 (eBioscience) following the manufacturer's protocol and then iv injected via tail vein into Mtb-infected WT or CD1d^{-/-} mice. Twenty hours later, mice were euthanized and lungs were removed and digested. Lung mononuclear cells were stained by surface and ICS protocols and paraformaldehyde fixed. Untransferred iNKT cells cultured in standard media were similarly treated to ascertain baseline activation. Flow cytometry gating strategies allowed for separation of endogenous versus transferred iNKT cells independent of tetramer staining by the presence of fluorescent dye.

Flow cytometry and ICS

Cells were first incubated with CD16/CD32 (FcBlock; BD Biosciences). Surface staining for in vitro experiments included antibodies for mouse CD69 (H1.2F3), CD25 (PC61), F4/80 (BM8), CD1d (1B1), and isotype controls (all from Biolegend). Surface staining of lung mononuclear cells included antibodies for mouse TCRβ (H57-597), CD69, CD3 (17A2), CD19 (6D5) and isotype controls (all from Biolegend). PBS-57-loaded and control PE- and APC-conjugated CD1d tetramers were provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility (Emory University Vaccine Center). After tetramer staining, ICS with antibodies specific for mouse GM-CSF (MP1-22E9; eBioscience) and IFNγ (XMG1.2; Biolegend) was performed following fixation with 4% paraformaldehyde and permeabilization with Perm/Wash buffer (BD Biosciences). Data were collected using FACSCanto (BD Biosciences) and analyzed with FlowJo (Tree Star, Inc.).

ELISA, Nitric Oxide, and Bioplex Immunoassays

Culture supernatants were filtered through 0.2 μm filter to remove any bacteria. IFNγ, IL-12p40, and GM-CSF ELISAs were done in accordance with the manufacturer's instructions (Biolegend), and absorbance was recorded at 450 nm on SoftMax Pro ELISA analysis software (Molecular Devices). Nitric oxide (NO) production was measured using the Griess reaction to detect nitrite, a stable breakdown product of NO, as described previously¹⁸. For size fractionation, Amicon Ultra-

15 Centrifugal Filter Units with 10kDa and 50kDa cutoff were used (EMD Millipore). Bioplex immunoassay was done in accordance with the manufacturer's instructions (BioRad).

Statistical analysis

Data was analyzed by one-way ANOVA (95% confidence interval) with Dunnett's post-test (for comparison against a single control) or Tukey post-test (for comparison between all conditions) or unpaired Student's t-test. Analysis was performed using GraphPad Prism software.

Acknowledgements

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Chapter 3: Antimicrobial activity of T cell-derived GM-CSF

This work is part of an unpublished and untitled manuscript with assistance from the following people: Claudio Nunes-Alves (University of Massachusetts Medical School, Worcester, MA), Girija Goyal and Glenn Dranoff (Dana-Farber Cancer Institute, Boston, MA).

ABSTRACT

There are several key T cell effector cytokines that have been shown to be critical for control of *Mycobacterium tuberculosis* (Mtb). These include IFN γ and TNF. To gain a more comprehensive understanding of what constitutes effective immunity, other protective T cell factors must be tested as well. We have identified GM-CSF as a protective cytokine produced by iNKT cells in response to Mtb infection. Here we test the contribution of T cell-derived GM-CSF to the Mtb immune response. We demonstrate that GM-CSF is produced in the lung during Mtb infection and both innate and adaptive T cell subsets in the lung produce GM-CSF over the course of infection. Preliminary adoptive transfer experiments reveal that T cell-derived GM-CSF contributes to antimicrobial control in vivo, despite having no direct effect on myeloid recruitment or global T cell function. In vitro experiments also demonstrate that the direct antimicrobial effect of GM-CSF on Mtb-infected macrophages enhances the direct effects of IFN γ at low cytokine concentrations. Together, these data establish GM-CSF as a protective T cell effector function during Mtb infection.

INTRODUCTION

In order for the host immune response to effectively control infection of *Mycobacterium tuberculosis* (Mtb), a strong T cell response is required¹. IFN γ has been identified as a key immune factor that can lead to inhibition and killing of Mtb through induction of nitric oxide², phagolysosomal fusion³, autophagy⁴, and Vitamin D receptor expression⁵. Although many studies have measured T cell production of IFN γ and many TB diagnostic tools rely on IFN γ from T cells for their readout, formal analysis has only recently been performed to show that IFN γ from CD4⁺ T cells is critical for bacterial control⁶. However, there is also evidence that T cells can control Mtb growth in vivo independently of IFN γ ^{7, 8}. It is likely that both IFN γ -dependent and independent pathways are important for complete immune control.

Apart from IFN γ , there are several other T cell effector functions that have been described to contribute to an antimicrobial response. TNF production plays an important role in controlling bacterial growth⁹. IL-17 has been shown to be effective early on in controlling Mtb and for effective granuloma formation, but also can have detrimental effect increasing immunopathology later during infection^{7, 10, 11}. In addition, CD8⁺ T cells have been shown to control infection through cytolytic activity^{12, 13}. Identifying additional T cell effector functions could help improve vaccine efforts and offer new immunotherapy targets.

GM-CSF is a T cell effector function that has been shown to be a factor in initiating autoimmune conditions like experimental autoimmune encephalomyelitis (EAE)^{14, 15}. GM-CSF has also been identified as a key protective cytokine during Mtb infection. GM-CSF treatment of human macrophages inhibits intracellular growth of Mtb and *M. avium*^{16, 17, 18}. We demonstrated in *Chapter 2* that iNKT cell production of GM-CSF contributes to their ability to restrict bacterial growth in vitro and that GM-CSF treatment of macrophages restricts Mtb growth in murine cells.

GM-CSF^{-/-} mice are highly susceptible to Mtb, with increased bacterial burden and decreased survival after virulent aerosol infection compared to WT mice^{19, 20}. GM-CSF is produced by many different cell types including leukocytes, epithelial cells and fibroblasts and is critical for the development and maturation of pulmonary macrophages. Interestingly, the absence

of this cytokine leads most dramatically to defects in lung function, while the effect on other organs is minimal²¹. Lack of GM-CSF leads to abnormalities in surfactant recycling and a pulmonary inflammatory disease resembling human pulmonary alveolar proteinosis (PAP). Overexpressing GM-CSF in epithelial cells reverses these lung abnormalities²² but the susceptibility to Mtb is not fully rescued, suggesting that GM-CSF production by other cells, perhaps T cells, may contribute to protection in mice^{19, 20}. Additionally, the presence of anti-GM-CSF autoantibodies that block GM-CSF function has been linked to susceptibility to both cryptococcal meningitis and pulmonary TB in otherwise healthy subjects indicating that GM-CSF may also participate in host defense against infection in people.

Here we demonstrate that GM-CSF is produced in the lung during Mtb infection and that both innate and adaptive T cell subsets produce GM-CSF at the site of infection. Through preliminary adoptive transfer experiments we demonstrate that T cell-derived GM-CSF contributes to antimicrobial control in vivo. Lastly, we show that the antimicrobial effect of GM-CSF on Mtb-infected macrophages is additive in combination with the direct effects of IFN γ . Together, these data reveal that GM-CSF is a protective T cell effector function during Mtb infection.

RESULTS

GM-CSF is produced during the normal immune response to Mtb

To determine the overall production of GM-CSF in the lung during *Mtb* infection, we measured GM-CSF protein in lung homogenate by Bioplex. We found an increase in total GM-CSF protein over the course of infection (*Figure 3-1A*) with a fold change of 1.97 ± 0.28 (mean \pm SEM) at 4 weeks, 5.02 ± 0.22 at 12 weeks, and 5.11 ± 0.11 at 24 weeks compared to naïve lungs (*Figure 3-1B*). This data shows that GM-CSF production accumulates over the course of infection and increases in the lung in a manner consistent with an adaptive immune response.

GM-CSF is produced by multiple T cell subsets during infection

Early work studying the pulmonary effects of GM-CSF showed that pulmonary epithelial cells could produce GM-CSF in culture²³. In addition, insertion of a GM-CSF transgene on a promoter expressed by type II lung epithelial cells (surfactant C) rescued the GM-CSF^{-/-} PAP phenotype²². Therefore, it has been assumed that epithelial cells were one of the main producers of GM-CSF in the lung. However, these studies did not address the dynamics of an immune response. Our previous work provided evidence that at least one T cell subset, iNKT cells, produced GM-CSF in the lung during infection (*Chapter 2*).

Therefore, we undertook a more comprehensive analysis of GM-CSF production by T cell subsets in the lungs of naïve and infected mice. We performed intracellular cytokine staining (ICS) analysis of lung samples at multiple time points post-infection in the presence of IL-2 and Brefeldin A without further stimulation. Separate staining panels were set up to assess 4 major T cell subsets, gated following doublet and auto-fluorescence exclusion: iNKT cells (TCR β ⁺ CD1d-tetramer⁺), $\gamma\delta$ T cells (CD3⁺ TCR β ⁻ TCR $\gamma\delta$ ⁺), CD4⁺ T cells (TCR β ⁺ CD4⁺ CD8⁻), and CD8⁺ T cells (TCR β ⁺ CD4⁻ CD8⁺). We found that all 4 cell types produced GM-CSF at some point during infection. A small but detectable number of iNKT cells and $\gamma\delta$ T cells produced GM-CSF early during infection. CD4⁺ T cells dominated the GM-CSF response by week 4, while CD8⁺ T cells did not start producing GM-CSF reliably until week 8 post-infection (*Figure 3-2A*). We then compared

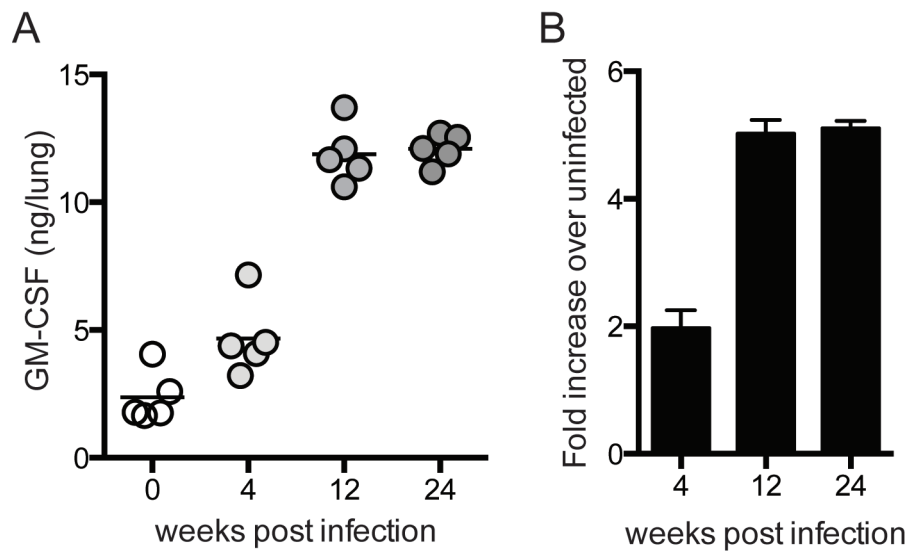


Figure 3-1. GM-CSF production in the lung increases over the course of Mtb infection.

GM-CSF measured by Bioplex immunoassay in lung homogenate at multiple weeks post aerosol infection with Erdman strain in WT C57Bl/6J mice. (A) GM-CSF protein normalized per lung. (B) Fold increase in GM-CSF protein at infection timepoints compared to naïve lung (0 weeks post infection). Data is from a single experiment at multiple timepoints with 5 mice per group.

this pattern of GM-CSF production to that of IFN γ production, where the response was dominated by CD4⁺ and CD8⁺ T cells starting at week 4 post-infection (*Figure 3-2B*). Interestingly, while there were very few double positive (GM-CSF⁺IFN γ ⁺) iNKT cells or $\gamma\delta$ T cells especially early during infection, there were many double positive CD4⁺ and CD8⁺ T cells by weeks 4 and up (*Figure 3-2C*).

The relative proportion of each T cell subset producing both GM-CSF and IFN γ over the course of infection was also analyzed (*Figure 3-3*). While the IFN γ response was clearly dominated by CD4⁺ T cells throughout the entire infection, GM-CSF production was more evenly split among the four T cell subsets analyzed. Both iNKT cells and $\gamma\delta$ T cells dominated the production of GM-CSF early during infection through week 3. CD4⁺ T cells made up the greatest proportion of GM-CSF⁺ T cells at week 4 and week 8 and CD8⁺ T cells made up the greatest proportion of GM-CSF⁺ T cells at late time points.

This data shows that both innate and adaptive T cell subsets produce GM-CSF as part of an immune response to Mtb infection. In addition, it demonstrates that GM-CSF and IFN γ production are regulated by different signals during infection. While the GM-CSF response has clear differences in kinetics compared to the IFN γ response, it is interesting to note that later during infection there are significant numbers of T cells producing both cytokines.

GM-CSF production by T cells is critical for control

Given that both innate and adaptive T cell subsets were found to produce GM-CSF during infection, we next determined whether GM-CSF production by T cells contributed to control of bacterial growth in vivo. We performed adoptive transfer experiments, transferring naïve T cells from either WT or GM-CSF^{-/-} mice that were isolated from spleen and lymph nodes, separated by magnetic bead selection, and then transferred into Rag^{-/-} hosts. These mice were then infected with Erdman strain of Mtb by low dose aerosol infection within 24 hours. After 4 weeks, lung and spleen were removed and analyzed for bacterial burden, pathology, and T cell cytokine production.

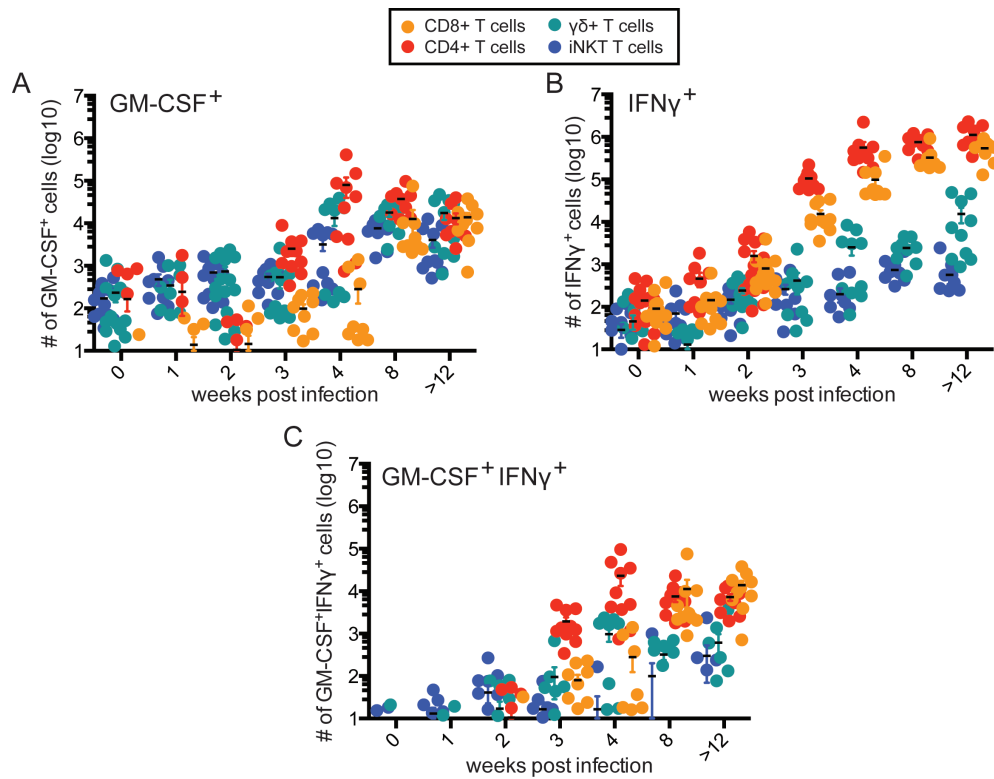


Figure 3-2. Multiple T cell subsets produce GM-CSF in the lung during Mtb infection.

Number of GM-CSF (A), IFN γ (B), and double producing (C) T cells isolated from the lung of WT C57Bl/6J after aerosol infection with Erdman strain and stained by ICS with no further stimulation and 4 hours of Brefeldin A incubation. iNKT cells (TCR β ⁺ CD1d-tetramer⁺), $\gamma\delta$ T cells (CD3⁺ TCR β ⁻ TCR $\gamma\delta$ ⁺), CD4⁺ (TCR β ⁺ CD4⁺ CD8⁻), CD8⁺ (TCR β ⁺ CD4⁻ CD8⁺). Data is compiled from 6 independent infections with at least 2 independent infections per time point.

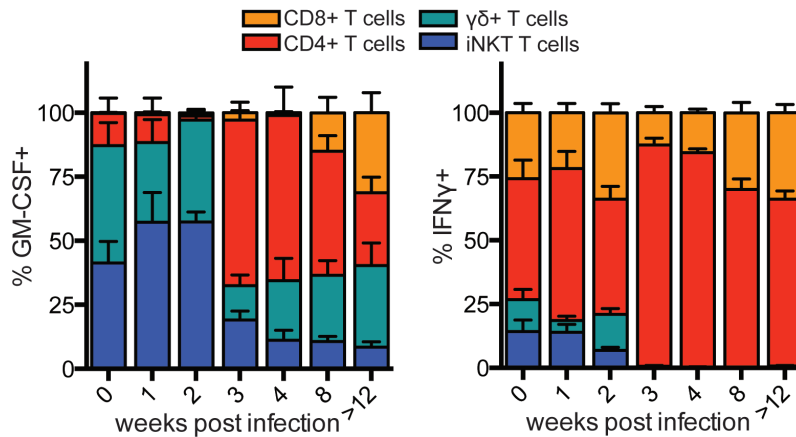


Figure 3-3. Relative proportion of cytokine production by T cell subsets over the course of infection.

Relative frequency of GM-CSF and IFN γ producing T cells from 4 major T cell subsets at each time point. Cells were isolated from the lung of WT C57Bl/6J after aerosol infection with Erdman strain and stained by ICS with no further stimulation and 4 hours of Brefeldin A incubation. iNKT cells (TCR β ⁺ CD1d-tetramer⁺), $\gamma\delta$ T cells (CD3⁺ TCR β ⁻ TCR $\gamma\delta$ ⁺), CD4⁺ (TCR β ⁺ CD4⁺ CD8⁻), CD8⁺ (TCR β ⁺ CD4⁻ CD8⁺). Data is compiled from 6 independent infections with at least 2 independent infections per time point.

We found that mice that received WT T cells had lower bacterial burden than mice that received GM-CSF^{-/-} T cells, evident by both CFU enumeration as well as histology samples (*Figure 3-4A and 3-4B*). This data suggests that GM-CSF production by T cells contributes to control of Mtb during in vivo infection.

The absence of T cell-derived GM-CSF does not significantly alter other features of the pulmonary immune response

Because we observed a difference in bacterial burden between mice that received the WT versus GM-CSF^{-/-} T cell transfer, we next determined whether this was due to differences in recruitment or differentiation of immune cell populations. We specifically focused on myeloid cell populations, because previous reports suggest that GM-CSF plays a critical role in the maturation and development of myeloid cells, especially dendritic cells. By flow cytometry, we analyzed the following myeloid cell populations in the lung using surface markers: polymorphonuclear leukocytes (PMN) (CD3⁻ Ly6G⁺ CD11b⁺), iNOS-expressing macrophages²⁴ (CD3⁻ CD11c⁺ CD11b⁺), dendritic cells (DC) (CD3⁻ CD11c⁺ CD11b⁻), and a smaller CD8α⁺ DC population (CD3⁻ CD8α⁺ CD11c⁺) (*Figure 3-5A*). Although there were small differences in the percentages of the myeloid populations between the two groups, these trends were not reproducible over multiple experiments. Percentages of CD4⁺ T cells (TCRβ⁺ CD4⁺ CD8⁻), CD8⁺ T cells (TCRβ⁺ CD4⁻ CD8⁺), iNKT cells (TCRβ⁺ CD1d-tetramer⁺) and γδ T cells (CD3⁺ TCRβ⁺ TCRγδ⁺) were also analyzed and there were also no major differences between groups (data not shown). This data suggests that the lack of T cell-derived GM-CSF does not significantly alter the recruitment or differentiation of inflammatory cell populations in the lung.

By ICS, GM-CSF and IFNγ production by CD4⁺ and CD8⁺ T cells in response to both peptide and anti-CD3/anti-CD28 stimulation were measured (*Figure 3-5B*). As expected, GM-CSF^{-/-} T cells did not produce any GM-CSF. This also served as a control for our antibody staining. However, we did not observe any differences in IFNγ production for either CD4⁺ or CD8⁺ T cells between the two groups. This indicates that any difference in bacterial growth produced by

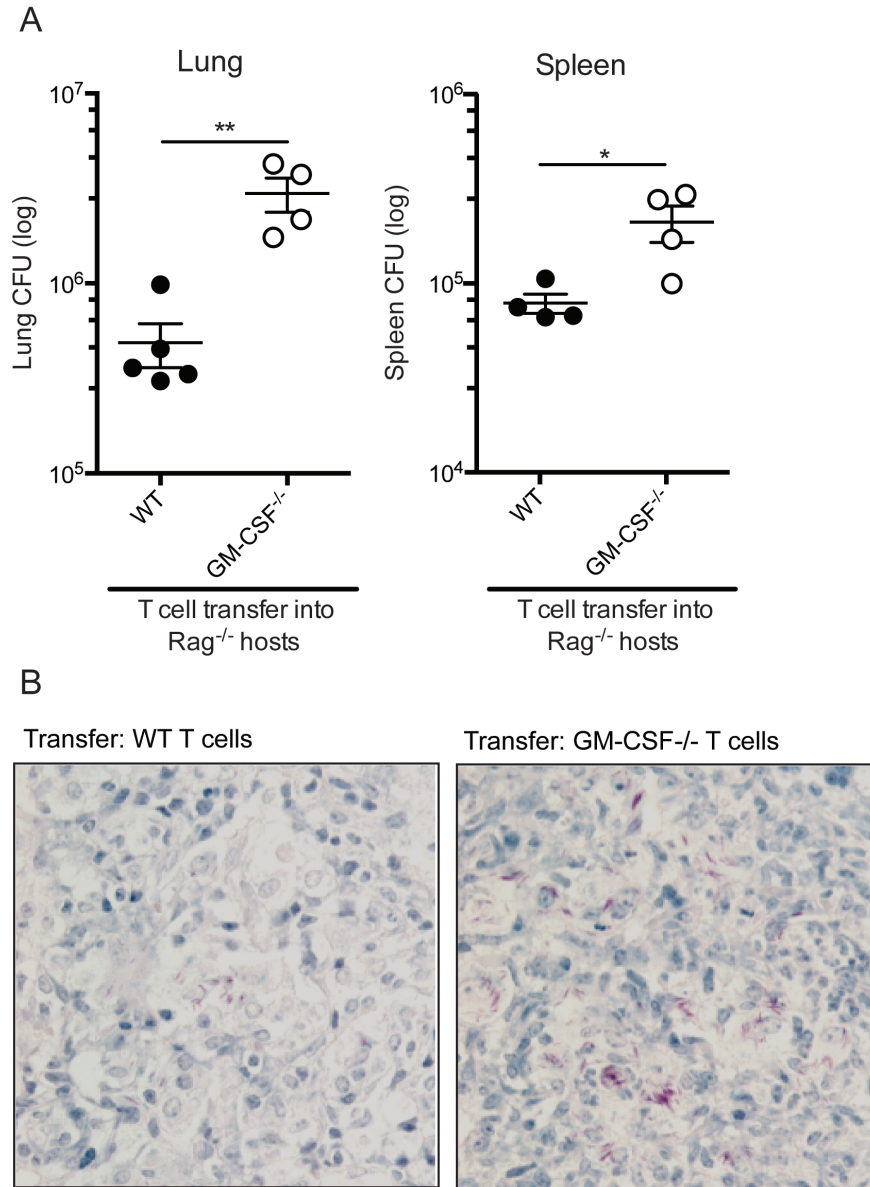


Figure 3-4. T cell derived GM-CSF is critical for control of Mtb growth.

WT and GM-CSF^{-/-} naïve T cells isolated from spleen and lymph nodes were transferred on d0 into Rag^{-/-} hosts, followed by low-dose aerosol infection with Erdman strain. (A) CFU measured in lung and spleen on d30 post-infection. (B) Representative histology samples from lung tissue fixed in formalin, paraffin-embedded and then stained with hematoxylin and eosin (H&E) and AFB. Student's unpaired t-test. * $p < .05$, ** $p < .01$. Data representative of two independent experiments.

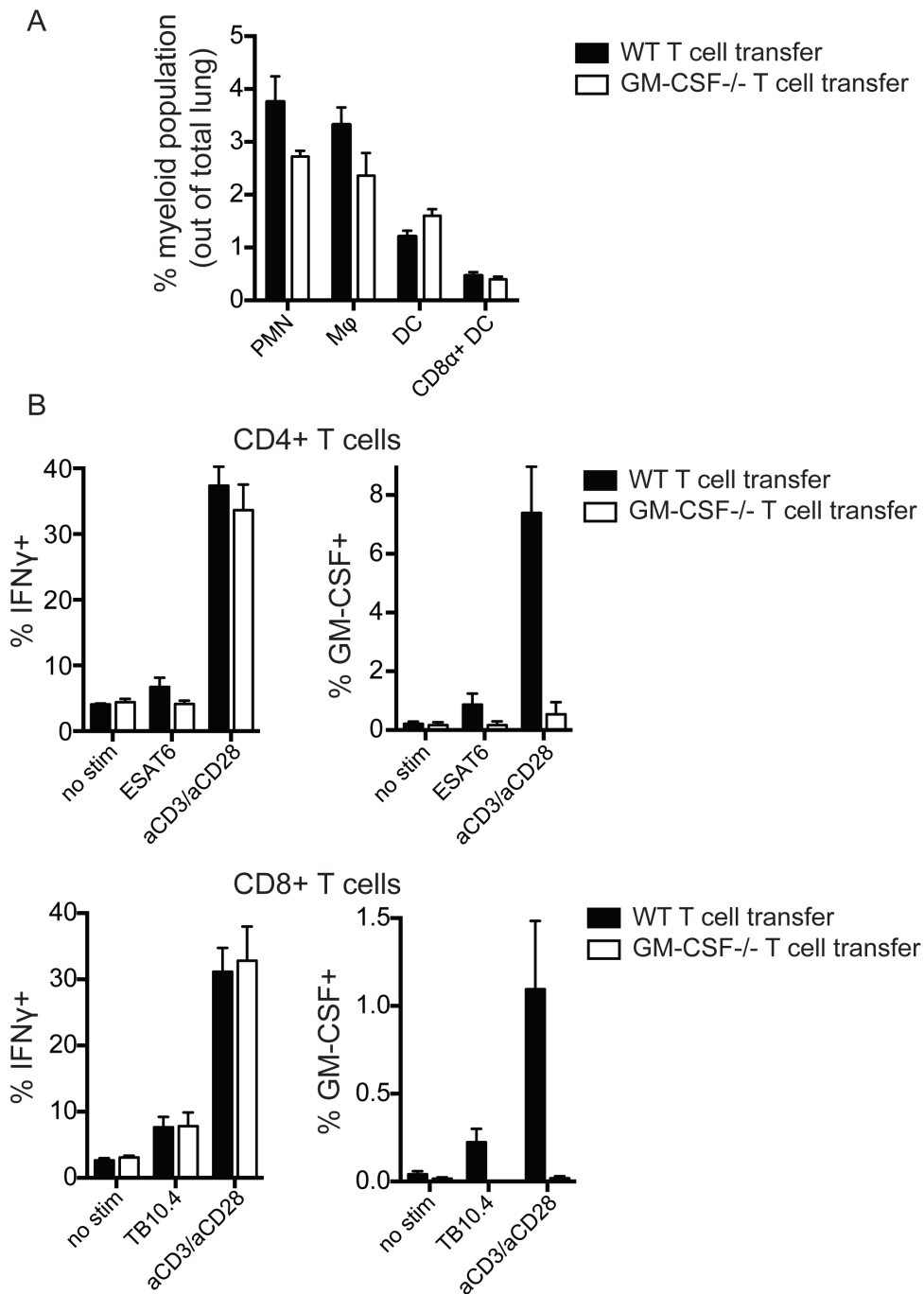


Figure 3-5. GM-CSF^{-/-} T cells still produce IFNγ.

30 days after transfer of WT and GM-CSF^{-/-} naïve T cells into Rag^{-/-} hosts and low-dose aerosol infection with Erdman strain, T cells were isolated from the lungs of infected mice. (A) By flow cytometry, the percentage of myeloid cell populations in the total lung were analyzed based on the following surface markers: PMN (CD3⁻ Ly6G⁺ CD11b⁺), Mφ (CD3⁻ CD11c⁺ CD11b⁺) cells, DC (CD3⁻ CD11c⁺ CD11b⁻), CD8α⁺ DC population (CD3⁻ CD8α⁺ CD11c⁺). (B) Cells were stimulated for 5 hours with peptides ESAT6 and TB10.4 and anti-CD3/anti-CD28 with Brefeldin A and IL-2. Intracellular cytokine staining for GM-CSF and IFNγ was performed. Error bars indicate mean ± SEM. Data representative of two independent experiments.

T cell-derived GM-CSF is unlikely to be the result of major changes in immune cell recruitment, overall T cell function, or IFN γ production more specifically.

GM-CSF and IFN γ have an additive antimicrobial effect

In vivo data suggested that the absence of T cell-derived GM-CSF led to an increase in overall bacterial burden, despite intact immune cell recruitment and an adaptive IFN γ response. ICS data in intact mice also demonstrated that during the adaptive response there were a significant proportion of CD4⁺ and CD8⁺ T cells that were GM-CSF⁺ IFN γ ⁺ double positive cells. We hypothesized that GM-CSF and IFN γ could be working together to directly limit bacterial growth. Utilizing our in vitro macrophage infection model, we tested whether the antimicrobial effects of GM-CSF and IFN γ could be augmented when combined (*Figure 3-6*). Both GM-CSF and IFN γ were individually able to inhibit Mtb growth at higher cytokine concentrations (such as 10 ng/ml). However, at lower concentrations of 0.1 ng/ml, the combination of GM-CSF and IFN γ led to a statistically significant decrease in CFU from the single cytokine treatment conditions. This indicates that especially at lower cytokine concentrations that are likely more representative of concentrations perceived by infected cells in the lung, GM-CSF and IFN γ appear to have an additive effect. Although the results do not reveal a clear synergistic effect between these cytokines, further investigation into the antimicrobial mechanism of GM-CSF may reveal mechanistic connections with IFN γ that will compel follow-up studies.

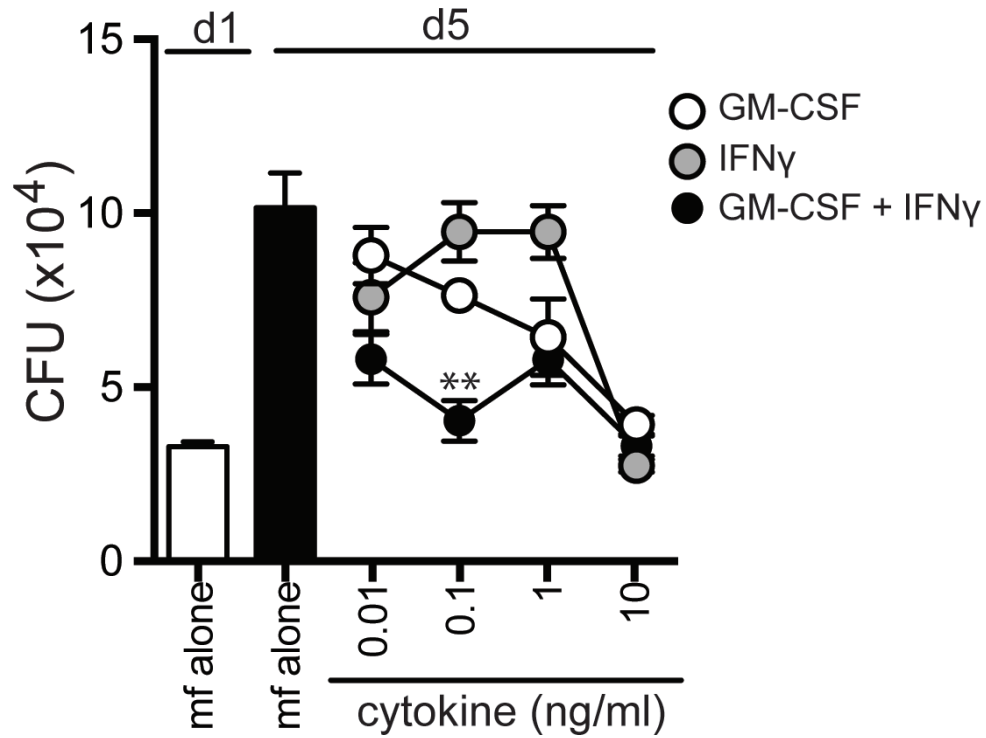


Figure 3-6. GM-CSF and IFN γ have an additive antimicrobial effect.

CFU assay for H37Rv-infected WT $m\phi$ with recombinant GM-CSF and/or IFN γ added on d1 post-infection. CFU were measured on d1 ($m\phi$ alone) and on d5 post-infection. Error bars indicate mean \pm SEM. **P < 0.01 (One way ANOVA with Dunnett's post-test, comparing combination treatment with individual cytokine treatment at each concentration.) Data are representative of two independent experiments with three replicates per condition.

DISCUSSION

Here we have shown that GM-CSF protein increases in the lung over the course of infection and can be produced by both innate (iNKT, $\gamma\delta$ T cells) and adaptive (TCR $\alpha\beta^+$ CD4 $^+$ and CD8 $^+$ T cells) T cell subsets in the lung in response to Mtb infection. Using an adoptive transfer model, we now have preliminary evidence that T cell-produced GM-CSF contributes to control of bacterial growth in vivo. Mice lacking GM-CSF-producing T cells have about a half of a log increase in CFU at four weeks post infection compared to mice with WT T cells. In vitro experiments demonstrated that GM-CSF can have a direct antimicrobial effect on Mtb-infected macrophages (*see also Chapter 2*) and here we show that this direct effect of GM-CSF can work in concert with IFN γ , especially at low cytokine concentrations. This suggests that GM-CSF and IFN γ may work together in the lung to control Mtb growth during the adaptive immune response, when T cells are producing both cytokines concurrently (*Figure 3-2C*).

There are at least two previous examples in the literature that describe GM-CSF production by human T cells in response to mycobacterial infection. The first study examined CD4 $^+$ CD1-restricted T cell lines derived from the lesions of leprosy patients that produced IFN γ and GM-CSF in response to sonicated *Mycobacterium leprae*²⁵. The second study analyzed T cells from the peripheral blood of children with either active or latent TB and found that CD4 $^+$ T cells produced GM-CSF in response to both Mtb and Mtb-antigens, but this population was not increased in active versus latent patients²⁶.

Establishing GM-CSF as a novel T cell effector function during Mtb infection identifies another component of the anti-mycobacterial T cell arsenal, which potentially could be utilized for host-directed therapeutics. This is critical because our current understanding of what immune factors need to be elicited for an effective vaccine response remains limited and is likely incomplete. While the current TB vaccine, bacilli Calmette-Guerin (BCG), is used to elicit immune protection in pediatric patients, its efficacy in adults has been limited. Therefore, many new vaccine candidates are now being tested that could induce sufficient protective and long-lasting immunity in adults. Several recent vaccines tested in clinical trials have had disappointing results,

including the prime-boost model using MVA85A, made up of a modified and replication defective vaccinia ankara virus expressing the Mtb antigen 85A, to boost initial BCG vaccination. While MVA85A was shown to induce strong IFN γ responses²⁷, the latest pediatric clinical trial did not show efficacy among infants, aged 4-6 months, when given as a booster to BCG vaccination²⁸. Although it did induce IFN γ , TNF, and IL-2 producing antigen-specific T cells, this did not correlate with protection over the two-year follow-up²⁸. Studies such as this one suggest that we still do not have a complete picture of effective T cell-induced Mtb immunity. Therefore, it is critical to identify new factors such as GM-CSF that may potentially be more informative measures of protection. A better understanding of how GM-CSF cooperates with other cytokines produced in the lung, including IFN γ and TNF, could further improve our understanding of immune dynamics during TB infection.

The therapeutic effect of GM-CSF has already been tested in several recombinant therapy studies. In a study of pulmonary *Streptococcus pneumoniae* infection in mice, orotracheal instillation of adenoviral vectors encoding GM-CSF led to an increase in host survival and a decrease in bacterial burden²⁹, a proof-of-principle study demonstrating that increased expression of GM-CSF in the lung can alter bacterial infection outcomes. More recently, using an i.t. Mtb infection model, a single dose of adenoviral-encoded GM-CSF prior to infection was shown to decrease the CFU of BALB/c mice by 2 weeks post-infection through the end of the experiment at 15 weeks³⁰. In a third study of i.v. Mtb infection, mice received intramuscular treatment of recombinant IL-2 and/or GM-CSF with and without standard isoniazid/rifampin immunotherapy. While treatment with recombinant GM-CSF alone had only a minor effect on bacterial burden in the H37Rv infection, a more dramatic effect (a 1.5 log reduction at 6 weeks) was observed when rGM-CSF was given in combination with rIL-2 and antibiotics for treatment of an MDR-TB OB35 strain infection where treatment with standard antibiotics alone failed³¹.

Interestingly, a human Phase II safety trial was conducted in Brazil for recombinant GM-CSF treatment of tuberculosis over a decade ago. Recombinant GM-CSF was injected subcutaneously twice a week as an addition to standard drug therapy (isoniazid/rifampin plus two

months of pyrazinamide). After four weeks of treatment with very low side-effects, clinical outcomes were equal between GM-CSF treatment and placebo, although the authors found a small but non-statistical trend towards faster time to negative culture in the treated patients³².

Taken together, these studies suggest that under the right treatment conditions, GM-CSF has potential as an immunotherapy for tuberculosis, including MDR-TB. Finding the most effective dosing, timing, and treatment routes are all critical studies that still need to be performed before any human trials should be attempted.

In the end, the short half-life of GM-CSF may restrict the efficacy of a recombinant GM-CSF therapeutic approach³³. However, this study suggests that there are various T cell subsets that could be stimulated to increase endogenous GM-CSF production in the lung. While IFN γ production is dominated by conventional CD4⁺ T cells, GM-CSF production was detected in all 4 subsets tested (iNKT cells, $\gamma\delta$ T cells, CD4⁺ T cells, and CD8⁺ T cells). Research on non-conventional T cells has found that these cell types can be strongly stimulated by non-Mtb antigens that still lead to increased survival and decreased bacterial burden in Mtb-infected animals. For iNKT cells, treatment with α GalCer was shown to increase survival in susceptible mouse strains³⁴ and synergized with standard antibiotic treatment³⁵. For $\gamma\delta$ T cells, stimulation with IL-2 and phosphoantigen led to significant expansion and trafficking of $\gamma\delta$ T cells into the lung and increased resistance to Mtb infection of non-human primates³⁶. While GM-CSF production was not measured, IL-2 and phosphoantigen treatment led to increases in both IFN γ production as well as perforin and granulysin expression. These studies hint at potential strategies to induce GM-CSF production early on in the lung.

MATERIALS AND METHODS

Ethics Statement

All mice were bred and maintained using standard humane animal husbandry protocols. All animal experiments were performed in accordance with relevant guidelines for the care and handling of laboratory animals and were approved by the Dana Farber Cancer Institute Animal Care and Use Committee (Animal Welfare Assurance number A3023-01) or by the Department of Animal Medicine of the University of Massachusetts Animal Care and Use Committee (Animal Welfare Assurance number A2420-13) under Public Health Service assurance of the Office of Laboratory Animal Welfare guidelines. Mice infected with *M. tuberculosis* were housed in a biosafety level 3 facility under specific pathogen-free conditions in Animal Biohazard Containment Suites (Dana Farber Cancer Institute, Boston, MA and University of Massachusetts, Worcester, MA).

Mice

C57BL/6J (WT) and B6.129S7-Rag1/J (Rag^{-/-}) mice were obtained from Jackson Laboratories. C57BL/6J GM-CSF^{-/-} mice were provided by Dr. Glenn Dranoff³⁷.

In vivo aerosol infections

In vivo infections were performed using virulent Mtb (Erdman strain). For each infection, a bacterial aliquot was thawed, sonicated twice for 10 s, and then diluted in 0.9% NaCl/0.02% Tween 80. A 15-ml suspension of *M. tuberculosis* was loaded into a nebulizer (MiniHEART nebulizer; Vortran Medical Technology) and mice were infected via the aerosol route with a nose-only exposure unit (Intox Products) and received ~50–100 CFU/mouse. Mice were euthanized by CO₂ inhalation and lungs were aseptically removed after perfusion of 10 ml of sterile RPMI into the right ventricle of the heart. Lung mononuclear cells were obtained by mechanical disruption using a gentleMACS dissociator (Miltenyi Biotec) followed by incubation in collagenase (Sigma-

Aldrich) for 30 mins at 37°C. Cells were isolated by forcing suspensions through a 70 µM cell strainer and then enumerated in 4% trypan blue with a hemacytometer. For ICS stimulations, cells were plated in a 96-well plate and incubated for 4-5 hours at 37°C with IL-2 either in the absence of stimuli or in the presence of TB10.4₄₋₁₁ peptide (10 µM; New England Peptide), ESAT6₁₋₂₀ peptide (10 µM; New England Peptide), or αCD3/αCD28 (1 ug/mL; BioLegend). Brefeldin A (GolgiPlug, BD Biosciences) was added to the cultures 1 hour after the addition of exogenous stimuli.

Adoptive T cell transfer

Naïve T cells were isolated from the spleen and lymph nodes of WT and GM-CSF^{-/-} mice and then separated using the Pan T cell Isolation kit (Miltenyi Biotec) following manufacturer's protocol. Magnetic separation was performed using LS columns (Miltenyi Biotec). 5x10⁶ T cells were then iv injected via tail vein into each Rag^{-/-} mouse. All mice were infected with Mtb via the aerosol route within 24 h of cell transfer.

Flow cytometry and ICS

Cells were first incubated with CD16/CD32 (FcBlock; BD Biosciences). Surface staining included antibodies for murine TCRβ (H57-597), TCRγδ (UC7-13D5), CD3 (17A2), CD19 (6D5), CD4 (RM4-5), CD8(53-6.7), CD25 (PC61), CD69 (H1.2F3), CD11b (M1/70), CD11c (N418) Ly6C (HK1.4), Ly6G (1A8) and isotype controls (all from Biolegend). Except for iNKT cell staining, ICS with antibodies specific for mouse GM-CSF (MP1-22E9; eBioscience) and IFNγ (XMG1.2; Biolegend) was performed in Perm/Wash buffer (BD Biosciences) following fixation and permeabilization with Fix/Perm buffer (BD Biosciences). PBS-57-loaded and control PE- and APC-conjugated CD1d tetramers were provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility (Emory University Vaccine Center). For iNKT cells, after tetramer staining, ICS with antibodies specific for mouse GM-CSF and IFNγ (see above) was performed following fixation with 4% paraformaldehyde and permeabilization with Perm/Wash buffer (BD

Biosciences). Data were collected using FACSCanto (BD Biosciences) or MACSQuant (Miltenyi Biotec) and analyzed with FlowJo (Tree Star, Inc.).

Macrophage isolation and culture

Thioglycollate (TGL)-elicited peritoneal macrophages were lavaged 4–5 days after 3% intraperitoneal TGL injection and then isolated by positive selection with CD11b microbeads and LS magnetic columns (Miltenyi Biotec). Purified cells were over 95% F4/80⁺ CD11b⁺, as determined by flow cytometry. Macrophages were seeded at 1×10^5 in 96-well culture plates in complete RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% fetal calf serum (HyClone).

Mtb in vitro culture and infection

H37Rv was grown and prepared as previously described³⁸. Bacteria was counted and added to macrophages at an effective multiplicity of infection (MOI) of 0.2 for CFU experiments for two hours. Cultures were washed three times to remove extracellular bacteria. Infected macrophages were cultured overnight and iNKT cells or other conditions were added on d1. For CFU measurement, cells were lysed with 1% Triton X-100/PBS and lysate from quadruplicate conditions were plated in serial dilutions on Middlebrook 7H10 agar plates (ThermoFisher Scientific), and cultured at 37°C for 21 days. Infected macrophages were treated with the following reagents: recombinant murine GM-CSF (Peprotech), and recombinant IFN γ (murine, Peprotech).

ELISA and Bioplex Immunoassays

Protein from lung homogenate was extracted using the Bio-Plex Cell Lysis Kit (Bio-Rad) and filtered through 0.2 μ m filter to remove any bacteria. The concentration of GM-CSF was measured using a mouse Bio-Plex cytokine assay in accordance with the manufacturer's instructions (Bio-Rad).

Statistical analysis

All data are represented as mean \pm SEM. Data was analyzed by one-way ANOVA (95% confidence interval) with Dunnett's post-test (for comparison against a single control) or unpaired Student's t-test. Analysis was performed using GraphPad Prism software.

Acknowledgements

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Chapter 4: Antimicrobial mechanism of GM-CSF

This work is part of an unpublished and untitled manuscript with assistance from the following people: Girija Goyal and Glenn Dranoff (Dana-Farber Cancer Institute, Boston, MA), Sarah Kinicki, Karin Green, Scott Schaffer, Kadamba Papavinasundaram, and Chris Sassetti (University of Massachusetts Medical School).

ABSTRACT

GM-CSF^{-/-} mice are highly susceptible to Mtb infection compared to WT mice, yet the direct antimicrobial function of GM-CSF has not yet been elucidated. We have now identified that GM-CSF is produced during Mtb infection by different T cell subsets. Here we characterize the direct antimicrobial effect of GM-CSF on Mtb-infected macrophages. We show that GM-CSF antimicrobial activity requires macrophage PPAR γ signaling. GM-CSF leads to an increase in neutral lipid content within macrophages and preliminary data reveal quantitative changes in lipid species. Utilizing an Mtb mutant that lacks cholesterol import machinery, we show that GM-CSF-mediated growth inhibition requires cholesterol uptake by the bacteria. Furthermore, perturbing Mtb lipid metabolism using chemical inhibitors and metabolic cofactors, preliminary data shows that GM-CSF may induce a cellular environment of metabolic stress for the bacteria. To better understand how lipid accumulation alters macrophage activity, infected macrophages were treated with oxLDL, inducing a “foamy macrophage” state, which also led to inhibition in Mtb growth. Overall, these results indicate that GM-CSF plays a role in regulating lipid metabolism that may contribute to limiting intracellular Mtb growth. Here we have identified a pathway by which pro-inflammatory cytokine signaling impacts cellular metabolism, altering an intracellular niche for pathogens to restrict rather than promote bacterial growth.

INTRODUCTION

GM-CSF^{-/-} mice are highly susceptible to Mtb infection^{1, 2}. Although GM-CSF is often regarded as a myeloid growth factor, GM-CSF^{-/-} mice have relatively normal steady-state myelopoiesis³. Instead, some of the susceptibility of GM-CSF^{-/-} mice is derived from dysfunction of pulmonary alveolar macrophages due to the build-up of surfactants and subsequent inflammation in the lung^{4, 5}. In the absence of GM-CSF signaling during fetal development, alveolar macrophages do not mature properly from fetal monocytes⁶. Rescuing the pulmonary alveolar proteinosis (PAP)-like phenotype with overexpression of GM-CSF by surfactant protein C-expressing epithelial cells reduces the susceptibility to Mtb aerosol infection but does not eliminate it^{1, 2}. This disparity in susceptibility points to a second role for GM-CSF independent of alveolar macrophage homeostasis.

In addition to animal model experiments, clinical data has shown that development of anti-GM-CSF neutralizing antibodies is associated with both cryptococcal meningitis and pulmonary tuberculosis⁷. Because these neutralizing antibodies develop over time, these patients likely experienced normal alveolar macrophage development early in life, again suggesting that Mtb susceptibility from the absence of GM-CSF may include a more direct pathway than just early lung development.

Several studies have examined the direct antimicrobial effect of GM-CSF during in vitro mycobacterial infections. Recombinant GM-CSF was found to restrict growth of *Mycobacterium avium* infection in human monocyte-derived macrophages (hMDM)^{8, 9} and Mtb infection in hMDM¹⁰. It was also shown to synergize with recombinant TNF^{8, 9}. While reactive oxygen species were detected after GM-CSF treatment of Mtb-infected hMDM⁸, none of the studies defined a mechanism of control.

GM-CSF is associated with inducing numerous macrophage functions including: production of reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS), increased FcR-dependent and independent phagocytosis, increased PGE₂ production, enhanced priming for IFN γ /LPS-induced TNF production, and cell survival^{11, 12, 13}. GM-CSF was also

recently shown to induce zinc sequestration, leading to enhanced control of intracellular *Histoplasma capsulatum* infection in macrophages¹⁴.

Due to the association of GM-CSF^{-/-} mice with dysregulated surfactant recycling, another macrophage function that has been linked to GM-CSF is regulation of lipid metabolism⁵. Interestingly, research on Mtb has also zeroed in on bacterial lipid metabolism as one cellular process where Mtb may be vulnerable to host perturbations by demonstrating that Mtb has many strategies in place to maintain a balance between nutrient availability and toxicity¹⁵. Transcriptional analysis of Mtb found significant changes in fatty acid and cholesterol metabolism genes regulated over the course of infection in macrophages, suggesting that Mtb may sense changes in nutrient availability and respond at the transcriptional level¹⁶. For example, Mtb is sensitive to the type of carbon sources it imports and utilizes host fatty acids to alleviate metabolic stress caused by the accumulation of toxic cholesterol and propionyl-CoA metabolic products as well as upregulates cholesterol degradation enzymes in response to increased cholesterol exposure^{17, 18}.

Because Mtb can utilize both host fatty acids and cholesterol^{17, 19}, lipid-laden “foamy” macrophages have been thought to be havens for bacterial growth. They are often found within Mtb lesions and granuloma structures and their formation in vitro can be induced by mycobacterial lipids and TLR stimulation^{20, 21}. In vitro induction of foamy macrophages leads to an increase in expression of Mtb genes associated with dormancy and stress responses as well as growth arrest of *M. avium* species^{19, 22, 23}. This suggests that Mtb responds to changes in lipid availability in its environment. Whether these changes are beneficial to the pathogen by providing additional nutrients or are beneficial to the host by creating a stressful metabolic environment is still not fully understood.

Here we address the antimicrobial function of GM-CSF and examine a role for GM-CSF in regulating host lipid metabolism. We show that GM-CSF antimicrobial activity requires PPAR γ signaling and that GM-CSF alters total neutral lipid content within the macrophage as well as the identity of the specific lipid species. Using a bacterial mutant that lacks cholesterol import

machinery, we demonstrate that the Mtb growth restriction induced by GM-CSF treatment is blocked when Mtb cannot take up cholesterol. Lastly, using oxLDL-induction of a foamy macrophage-like phenotype, we validate that increased neutral lipid content leads to inhibition of bacterial growth in our model.

RESULTS

Recombinant GM-CSF restricts growth of Mtb

As demonstrated in Chapter 2, stimulation of thioglycollate-elicited peritoneal macrophages with recombinant GM-CSF leads to restriction in Mtb growth in a dose dependent manner (*Figure 2-18*). In order to investigate the antimicrobial mechanism of GM-CSF, a comprehensive analysis of multiple dose-titration experiments was conducted. The % CFU reduction was calculated for 10 independent experiments with GM-CSF doses between 0.01-10 ng/ml (*Figure 4-1A*). A striking dose-dependent effect was found with the % CFU reduction calculated as $25.8 \pm 3.5\%$ for 0.01 ng/ml, $43.8 \pm 5.8\%$ for 0.1 ng/ml, $63.8 \pm 5.0\%$ for 1 ng/ml and $70.3 \pm 5.3\%$ for 10 ng/ml (mean \pm SEM).

Recombinant GM-CSF has previously been reported to inhibit the growth of mycobacterium in hMDM^{8,9,10}. We confirmed these results by infecting primary monocyte-derived macrophages, from 4 independent healthy donors (*Figure 4-1B*). Although we observed variability among the different donors, GM-CSF inhibited bacterial growth to some degree in all cases, confirming that the antimycobacterial effect of GM-CSF pertained to both murine and human macrophages.

GM-CSF antimicrobial activity requires PPAR γ signaling in macrophages

GM-CSF is reported to have many different effects on macrophages including inducing phagocytosis, nitric oxide production, reactive oxygen species, and cell survival and proliferation. We examined many of these functions after GM-CSF stimulation and did not find evidence for GM-CSF altering these outcomes, apart from an increase in cell survival. This data is compiled in *Appendix I*.

To identify the antimicrobial mechanism of GM-CSF, we narrowed down our investigation by examining transcription factors that might be required for GM-CSF mediated control of Mtb growth within macrophages. While PPAR γ is not essential for macrophage development, it plays an important role in lipid recycling and surfactant catabolism, especially in alveolar

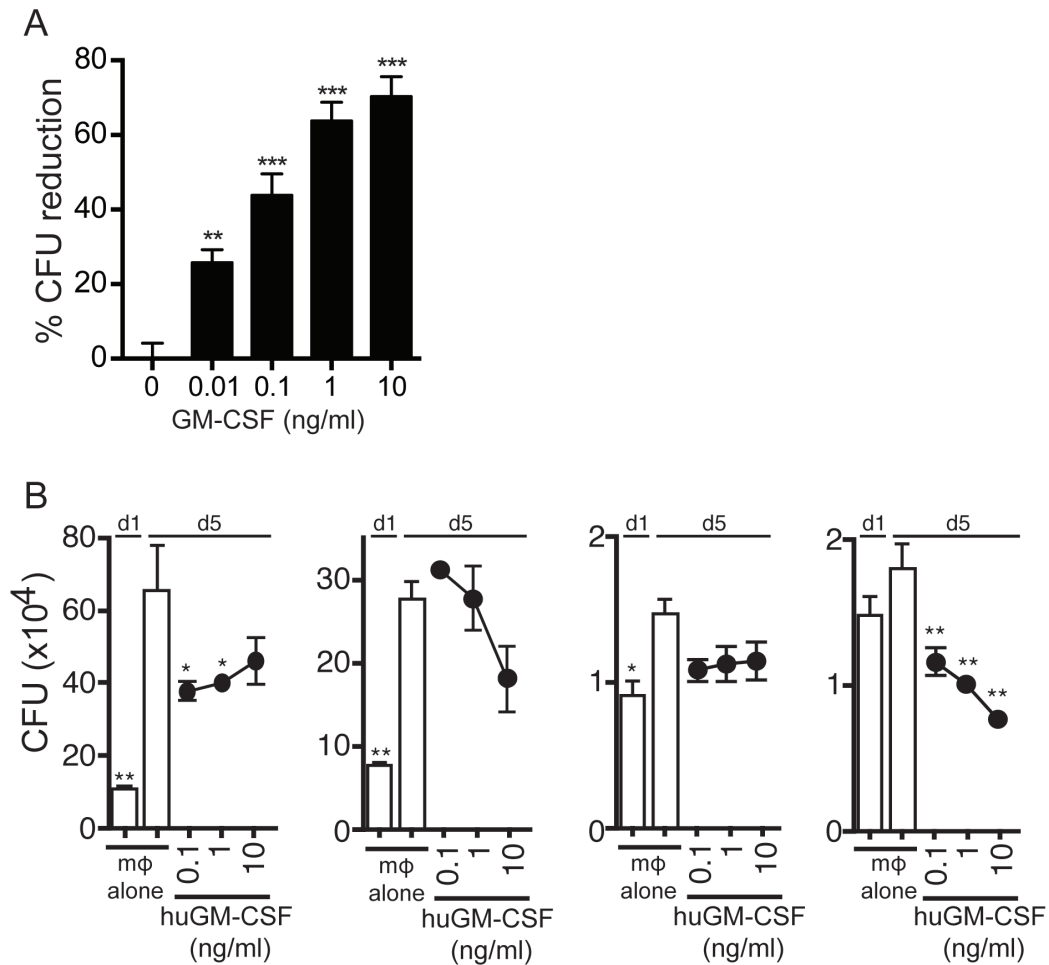


Figure 4-1. Recombinant GM-CSF restricts growth of Mtb.

(A) % CFU reduction calculated from CFU assays for H37Rv-infected WT murine mφ with recombinant GM-CSF (0.01-10 ng/ml). (B) CFU assay for H37Rv-infected human monocyte-derived macrophages (hMDM) with human recombinant GM-CSF (0.1-10 ng/ml). Error bars indicate mean \pm SEM. *P < .05, **P < 0.01, ***P < .001 (One-way ANOVA with Dunnett's post-test, compared to d5 untreated mφ). Data are compiled from 10 independent experiments (A) or from four independent experiments (B).

macrophages^{24, 25, 26, 27}. PPAR γ has specifically been linked to GM-CSF function through the rescue of the pulmonary alveolar proteinosis (PAP)-like phenotype in GM-CSF^{-/-} animals with lentiviral-directed overexpression of PPAR γ , leading to net cholesterol efflux and reduction of lipid accumulation in alveolar macrophages²⁸.

H37Rv-infected WT and PPAR γ ^{-/-} (PPAR γ ^{fl/fl}; LysM-cre) macrophages were treated with recombinant GM-CSF. While GM-CSF led to inhibition in bacterial growth in WT macrophages, no inhibition was observed for PPAR γ ^{-/-} macrophages (*Figure 4-2A*). However, IFN γ /TNF treatment, used here as a positive control for growth restriction, led to inhibition in both macrophages types. When % CFU reduction was calculated for 4 independent experiments, there was a statistically significant difference for the CFU reduction between WT and PPAR γ ^{-/-} macrophages for all GM-CSF doses between 0.01-10 ng/ml (*Figure 4-2B*). Similar results were found for comparisons between PPAR γ ^{-/-} (PPAR γ ^{fl/fl}; LysM-cre) and PPAR γ ^{fl/fl} control (PPAR γ ^{fl/fl}; no cre expression) macrophages (data not shown). This data suggests that PPAR γ signaling is involved in the antimicrobial effector pathway stimulated by GM-CSF in Mtb-infected macrophages.

GM-CSF alters gene expression of PPAR γ and downstream target genes

Given that GM-CSF appears to require PPAR γ expression in macrophages to restrict Mtb growth, we next determined whether GM-CSF was altering the gene expression of PPAR γ as well as several other downstream gene targets of PPAR γ . Previous research examining PPAR γ signaling in alveolar macrophages has identified that PPAR γ regulates expression of LXR α and LXR β expression that in turn regulates cholesterol efflux transporters including ABCA1, ABCG1, and ApoE²⁶, expression of receptors for modified LDL like CD36 and SR- β 1, and negatively regulates pro-inflammatory NF- κ B signaling^{29, 30}.

Preliminary quantitative RT-PCR analysis was performed on GM-CSF treated uninfected and H37Rv-infected macrophages. Surprisingly, GM-CSF treatment led to a modest reduction in gene expression of *Pparg*, *Lxra*, *Lxrb*, and *ApoE*, with an increase in *iNOS* expression that is consistent with an overall reduction in the PPAR γ signaling pathway (*Figure 4-3*). Although an

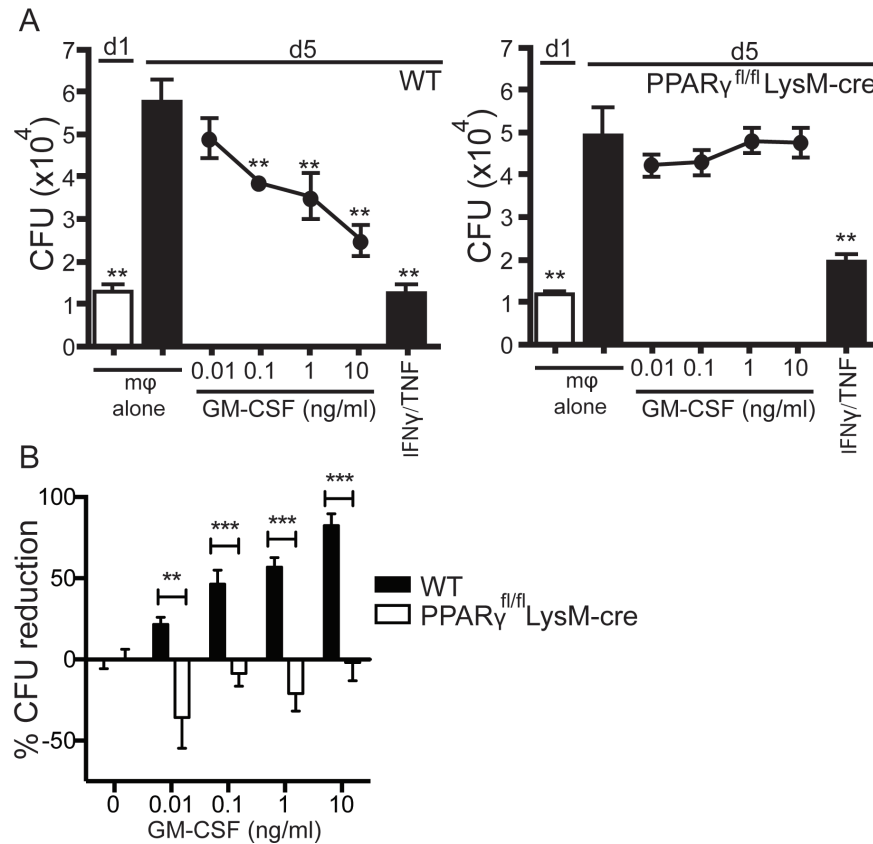


Figure 4-2. GM-CSF antimicrobial activity requires PPAR_γ signaling in macrophages.

CFU assay on d1 and d5 post-infection (A) and % CFU reduction calculated from CFU assays (B) for H37Rv-infected WT and PPAR_γ^{fl/fl}; LysM-cre mφ with recombinant GM-CSF (0.01-10 ng/ml). Recombinant IFN_γ (10 U/ml) and TNF (10 ng/ml) used for positive control. Error bars indicate mean ± SEM. **P < 0.01, ***P < .001 (One-way ANOVA with Dunnett's post-test, compared to d5 untreated mφ (A) and multiple Student's t-tests (B)). Data are representative of or compiled from 4 independent experiments.

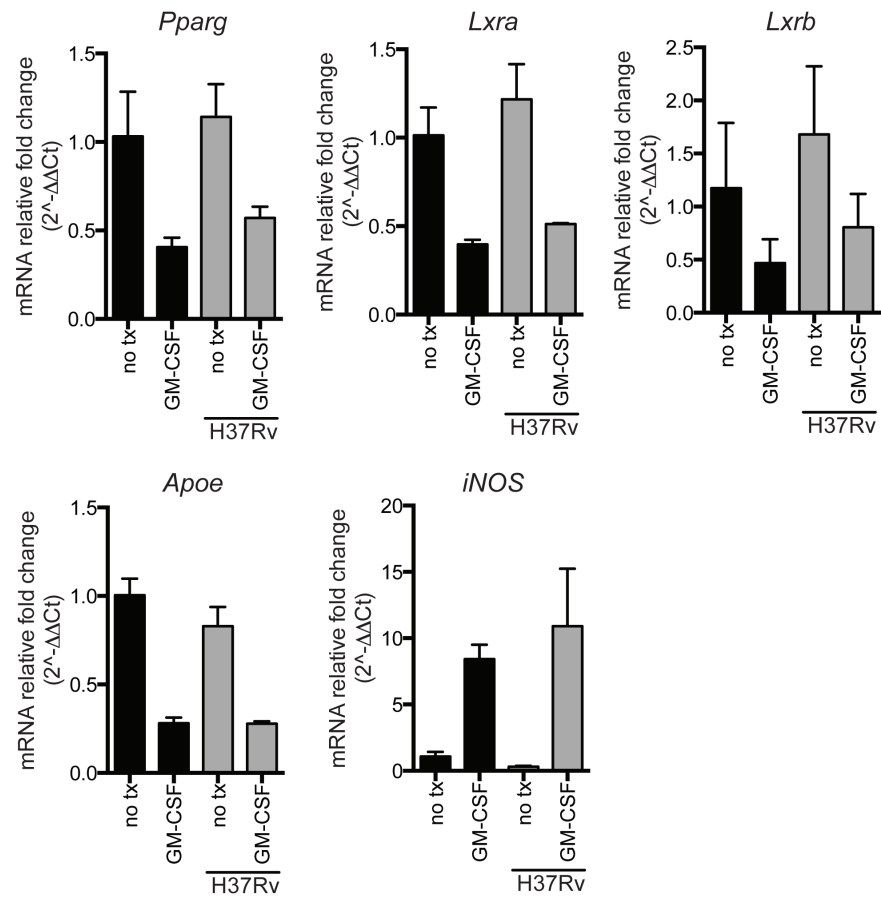


Figure 4-3. GM-CSF alters gene expression of PPAR γ and downstream target genes.

Quantitative RT-PCR analysis of *Pparg*, *Lxra*, *Lxrb*, *Apoe*, and *iNOS* mRNA in WT uninfected or H37Rv-infected m ϕ treated with or without GM-CSF (10 ng/ml) for 24 hours (except for *iNOS*, measured at 48 hours). Results were calculated by the comparative Ct method relative to HPRT (internal control gene for hypoxanthine-guanine phosphoribosyltransferase) and relative to RNA levels in uninfected, untreated m ϕ . Error bars indicate mean \pm SEM. Data are representative of 3 independent experiments.

increase in *iNOS* gene expression was observed, no increase in nitric oxide was detected in response to GM-CSF stimulation (see Appendix I, Supplemental Figure 1). This trend was independent of Mtb infection, observed for both uninfected and infected macrophages. Despite several reports that PPAR γ expression is induced during Mtb infection^{31, 32, 33}, this was not observed in this system. We also found two genes (*Abcg1*, *Abca1*) that are downstream of PPAR γ signaling that either had very little change in expression after GM-CSF treatment or inconsistent changes between experiments that did not seem to fit the overall signaling pattern observed for the other genes (data not shown). Overall, this data demonstrates that GM-CSF does not upregulate PPAR γ gene expression and may in fact be downregulating PPAR γ signaling, leading to a decrease in cholesterol efflux.

GM-CSF increases neutral lipid content in a PPAR γ -independent manner

Due to the changes in cholesterol efflux receptor gene expression by GM-CSF stimulation and the requirement for PPAR γ signaling for inhibition in Mtb growth by GM-CSF, we next determined whether GM-CSF changed the neutral lipid content of macrophages and whether this change was PPAR γ -dependent.

To evaluate changes in total lipid content in the cell after GM-CSF treatment, macrophages were incubated with a commonly used neutral lipid dye: 4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene (BODIPY 493/503). By flow cytometry, GM-CSF treatment of either uninfected or H37Rv-infected macrophages led to an increase in neutral lipid content, as measured by BODIPY fluorescence (FITC MFI) (Figure 4-4A). A similar result was found using a second neutral lipid dye, Oil Red O (data not shown). Neutral lipid staining was also verified by widefield microscopy to ensure that BODIPY was marking small puncta within the macrophages, as expected (Figure 4-4B). This increase in neutral lipid content did not appear to be PPAR γ -dependent, as this trend was observed for both the PPAR γ -floxed control (PPAR $\gamma^{\text{fl/fl}}$) and PPAR $\gamma^{-/-}$ (PPAR $\gamma^{\text{fl/fl}}$; LysM-cre) macrophages (Figure 4-4C).

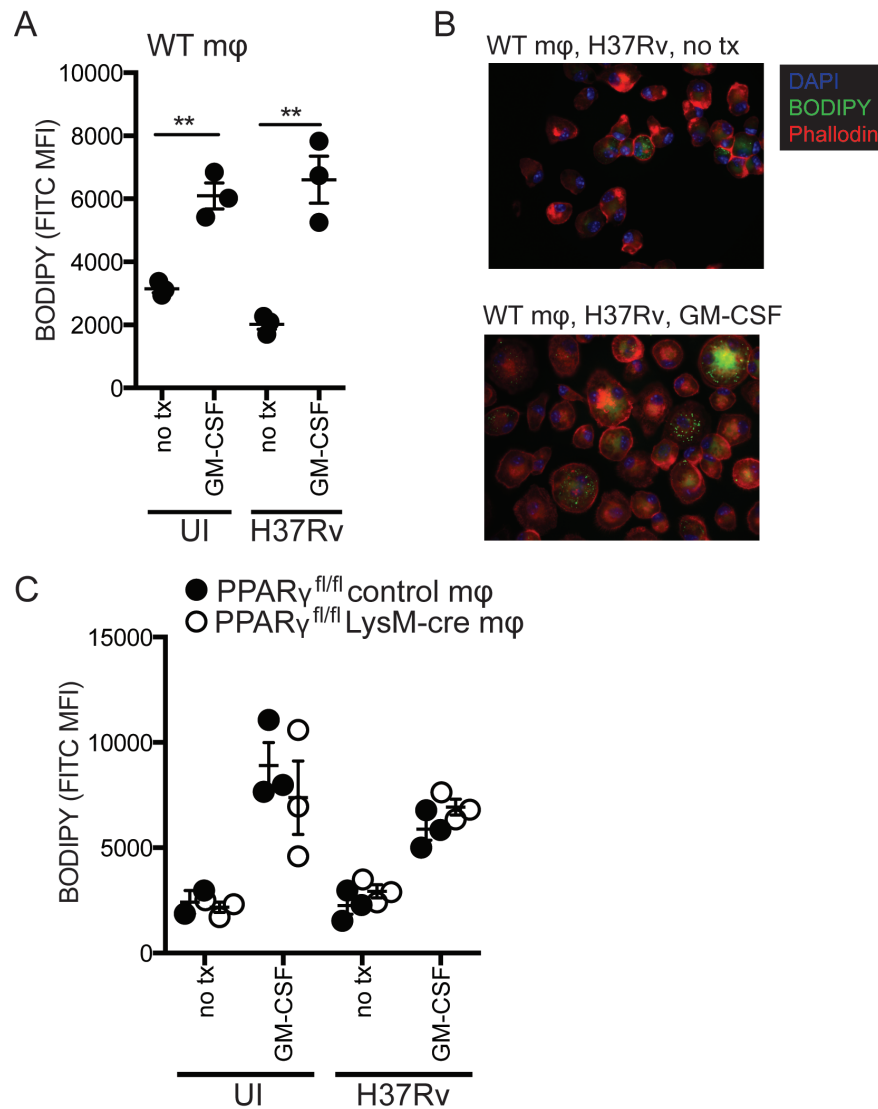


Figure 4-4. GM-CSF increases neutral lipid content in a PPAR γ -independent manner.

Recombinant GM-CSF (10 ng/ml) was added to uninfected or H37Rv-infected WT (A, B) and PPAR $\gamma^{fl/fl}$ control and PPAR $\gamma^{fl/fl}$; LysM-cre mφ (C) 1 day post-infection. After 4 days, cells were stained with BODIPY 493/503 for 20 minutes at 37°C and then fixed. (A, C) FITC MFI was determined by flow cytometry. (B) Representative images from widefield microscopy with BODIPY (green), DAPI nuclear (blue), and Phalloidin/F-actin (red) staining in fixed macrophages. Error bars indicate mean \pm SEM. **P < .01 (Unpaired student's t-test) (A). No difference in BODIPY MFI between two macrophage strains for any condition. (Multiple student's t-test) (C). Data are representative of 2 independent experiments.

Both the flow cytometry and microscopy data suggest that GM-CSF induces an overall increase in neutral lipids in macrophages, yet this effect is not specifically regulated by the PPAR γ signaling pathway.

GM-CSF induces qualitative and quantitative changes in macrophage neutral lipid content

Next, we further evaluated the changes in the types of neutral lipids found within GM-CSF treated macrophages by utilizing liquid chromatography-mass spectrometry analysis. Neutral lipids extracted from cell lysates from uninfected WT, PPAR $\gamma^{fl/fl}$; LysM-cre, and PPAR $\gamma^{fl/fl}$ control macrophages untreated or stimulated with GM-CSF were analyzed using positive ion electrospray mass spectrometry. Total amounts for each lipid family were calculated by comparing peak intensities of the samples to lipid standards spiked into each sample (see Materials and Methods). Although total neutral lipid extract weights were not measured due to low sample amounts, 10×10^6 macrophages were plated for each sample and the same percentage of each sample was analyzed so that the samples could be compared. In addition, we found the summed peak intensities (extracted ion chromatogram, XIC) for all samples were very similar (*Figure 4-5A*).

The data showed that total triacylglycerides increased and total ceramides decreased in response to GM-CSF (*Figure 4-5B*). In addition, GM-CSF induced changes in total free cholesterol and cholesterol esters. These preliminary results showed that changes in cholesterol content might be PPAR γ -dependent, although further repeat experiments will need to be performed to verify these trends. Overall, these data suggest that GM-CSF induces both qualitative and quantitative changes in the neutral lipid content of macrophages.

GM-CSF antimicrobial activity requires cholesterol uptake by Mtb

Measuring changes in total neutral lipid content (*Figure 4-4*) and qualitative changes in the lipid species (*Figure 4-5*) in GM-CSF-stimulated macrophages helps to reveal GM-CSF-induced host metabolic changes. However, in addition to evaluating the effect of GM-CSF on host

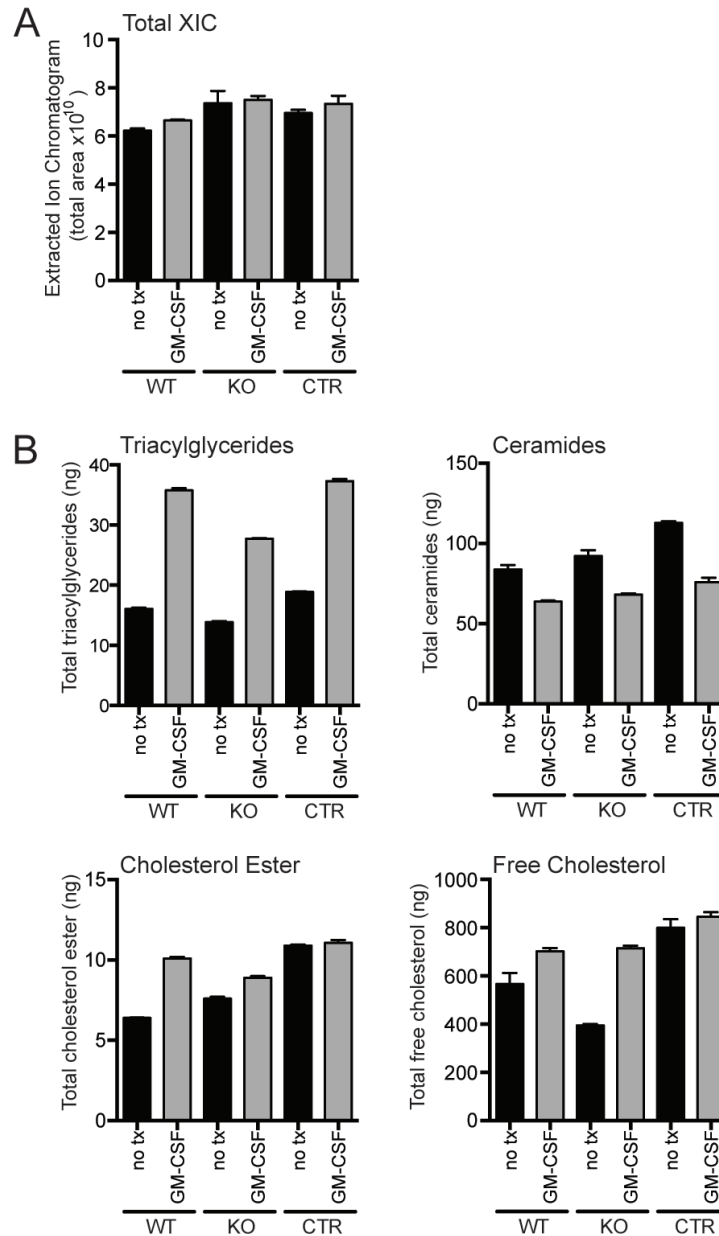


Figure 4-5. GM-CSF induces qualitative and quantitative changes in macrophage neutral lipid content.

Uninfected WT, $PPAR\gamma^{fl/fl}$, $LysM\text{-}cre$ (KO), and $PPAR\gamma^{fl/fl}$ control (CTR) m ϕ were untreated or stimulated for 4 days with 10 ng/ml GM-CSF, washed, scraped, and flash frozen. Neutral lipid extraction was performed on the cell extracts and samples were run through LC-MS. (A) Total extracted ion chromatogram of each sample. (B) Using lipid standards spiked into the samples, ng amounts of total triacylglycerides, ceramides, free cholesterol, and cholesterol esters were calculated for each sample. Data are representative of a single experiment, with technical triplicates.

pathways, we decided to survey the host-pathogen interface using Mtb mutants that lack genes for particular nutrient uptake or metabolism. Mtb relies upon a balance of carbon sources within a macrophage and alterations in this balance can affect bacterial growth^{17, 34}. Given the data above that GM-CSF increases the amount of neutral lipids in the macrophage and induces changes in cholesterol ester and free cholesterol species, we hypothesized that GM-CSF might induce cholesterol toxicity in Mtb and this might explain the reduction in bacterial growth that we observe after GM-CSF.

We utilized an Mtb mutant strain, $\Delta mce4$, that lacks cholesterol import machinery³⁵ to test whether alterations in cholesterol metabolism play a role in the antimicrobial effect of GM-CSF. Interestingly, this strain has been tested both for in vitro macrophages infections and in vivo murine infections^{35, 36}. The $\Delta mce4$ strain does not have a growth defect in WT macrophage infections or during acute infection in vivo, yet its growth is severely diminished in IFN γ -activated macrophages and chronic infection at late timepoints in vivo compared to WT Mtb. These results suggest that while Mtb does not require cholesterol utilization under all conditions, dynamics during host infection changes Mtb's requirement for cholesterol uptake. This is additional evidence that the host may have the ability to alter nutrient availability to Mtb over the course of infection.

When macrophages infected with WT H37Rv were treated with recombinant GM-CSF, it led to a reduction in Mtb growth, as observed previously (*Figure 4-6*). However, when macrophages infected with $\Delta mce4$ H37Rv were treated with recombinant GM-CSF, no inhibition in Mtb growth was measured, despite adequate growth restriction by the positive control, IFN γ /TNF. This result suggests that cholesterol import is required for the antimycobacterial action of GM-CSF.

GM-CSF inhibits the ability of Mtb to overcome 3-NP-mediated propionyl-CoA toxicity

The results from the $\Delta mce4$ H37Rv strain reveal that GM-CSF may be forcing the bacteria to import cholesterol. Mycobacterial cholesterol metabolism has been studied by several

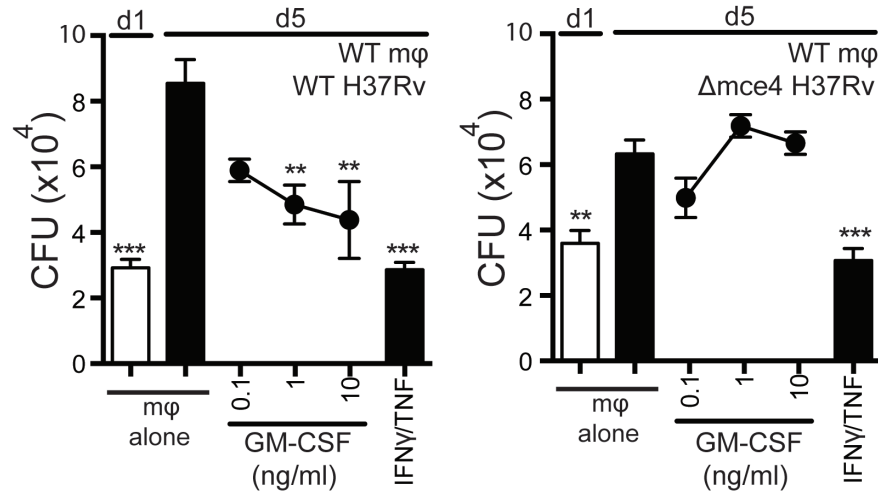


Figure 4-6. GM-CSF antimicrobial activity requires cholesterol uptake by Mtb.

CFU assay d1 and d5 post-infection for WT H37Rv (*left*) and Δmce4 H37Rv-infected (*right*) WT mφ with recombinant GM-CSF (0.1-10 ng/ml). Recombinant IFNγ (10 U/ml) and TNF (10 ng/ml) used for positive control. Error bars indicate mean ± SEM. **P < 0.01, ***P < .001 (One-way ANOVA with Dunnett's post-test, compared to d5 untreated mφ). Data are representative of 2 independent experiments.

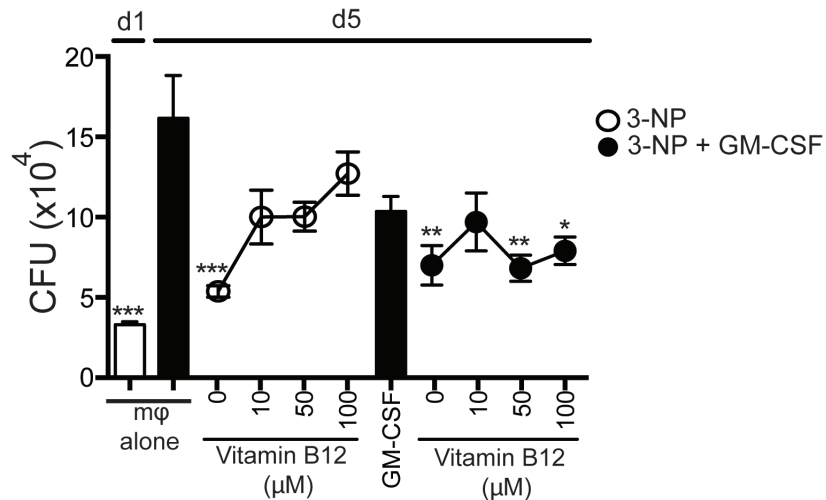


Figure 4-7. GM-CSF inhibits the ability of Mtb to overcome 3-NP-mediated propionyl-CoA toxicity.

CFU assay d1 and d5 post-infection with H37Rv-infected WT mφ treated on d1 with 3-nitropropionate (3-NP) (0.25 mM) to inhibit Mtb growth through cholesterol toxicity, rescued with co-treatment with Vitamin B12 (10, 50, 100 μM). Recombinant GM-CSF (10 ng/ml) added in addition. Error bars indicate mean ± SEM. *P < .05, **P < 0.01, ***P < .001 (One-way ANOVA with Dunnett's post-test, compared to d5 untreated mφ). Data is representative of two independent experiments.

groups and many of the metabolic intermediates and the regulating enzymes have been worked out^{15, 37}. One consequence of cholesterol metabolism that has been observed in mycobacteria is the resulting toxicity from propionyl-CoA pools. Multiple pathways of propionyl-CoA catabolism have been identified in Mtb that relieve this metabolic stress^{17, 34}.

Previously, 3-nitropropionate has been used as a chemical inhibitor of the methylisocitrate lyase and isocitrate lyase (MCL/ICL) pathways. An enzyme encoded by the Mtb gene *icl1* regulates these pathways. A Mtb $\Delta icl1$ mutant strain has been shown to have growth defects when cultured in cholesterol-containing media, even the presence of other carbon sources³⁸. Similarly, Mtb growth in the presence of 3-NP is severely diminished. Growth can be rescued with the addition of Vitamin B12, which activates the methylmalonyl pathway (MMP) converting propionyl-CoA into succinyl-CoA, relieving propionyl-CoA toxicity³⁴. Mtb cannot synthesize Vitamin B12 on its own, which is why exogenous Vitamin B12 supplementation activates this pathway.

As previously reported, when macrophages were infected with WT H37Rv and treated with 3-NP, growth of Mtb was dramatically inhibited (*Figure 4-7*). This growth defect was rescued in a dose-dependent manner with the addition of Vitamin B12. However, in the presence of recombinant GM-CSF, rescue by Vitamin B12 was inhibited. It is possible that cholesterol metabolism-independent effects of GM-CSF could explain this result. However, in combination with the other data collected, this result is consistent with the model that GM-CSF generates an excess of propionyl-CoA toxicity that the doses of Vitamin B12 used in this experiment were not enough to relieve the metabolic stress. Further follow-up studies are necessary to validate this interaction. This result suggests that GM-CSF induces mycobacterial metabolic stress through alterations in host metabolism.

oxLDL treatment leading to a foamy macrophage phenotype inhibits Mtb growth

The fate of Mtb after increased lipid content within host macrophages has been contemplated ever since it was observed that foamy macrophages, filled with lipid droplets,

accumulate within granulomas during chronic infection of tuberculosis²¹. The increase in cholesterol content within the infected macrophage by GM-CSF appeared to produce a similar host environment. While foamy macrophages have long been associated with disease severity and increased bacterial burden, there is little evidence that foamy macrophages themselves are directly beneficial or harmful to the host response^{19, 39}.

To directly address whether a “foamy” macrophage phenotype alters Mtb growth within macrophages, we fed macrophages oxidized low-density-lipoprotein (oxLDL)⁴⁰ after initial infection with H37Rv. Macrophages treated with oxLDL had significantly decreased bacterial burden compared to untreated macrophages by d5 and d7 post-infection (*Figure 4-8A*). This effect was also observed with resident peritoneal macrophages (*Figure 4-8B*). Measured by BODIPY staining, oxLDL treatment led to an increase in neutral lipid content, regardless of infection status of the macrophage (*Figure 4-8C*).

Given that oxLDL treatment leads to an increase in cholesterol, cholesterol esters, and triacylglyceride species, we next tested whether oxLDL treatment would lead to an inhibition in bacterial growth in the absence of Mtb cholesterol import. Similar to the results with rGM-CSF, oxLDL treatment did not inhibit growth of Mtb when the Mtb strain ($\Delta mce4$) lacked the ability to take up cholesterol (*Figure 4-8D*).

These results showed that an increase in neutral lipid content by a GM-CSF-independent mechanism also led to restriction of bacterial growth. This is additional evidence that alterations in host cellular metabolism can impact growth of intracellular Mtb and that this may be the antimicrobial mechanism of GM-CSF. Given the complexity of these metabolic pathways, further experiments will need to be performed to verify these findings.

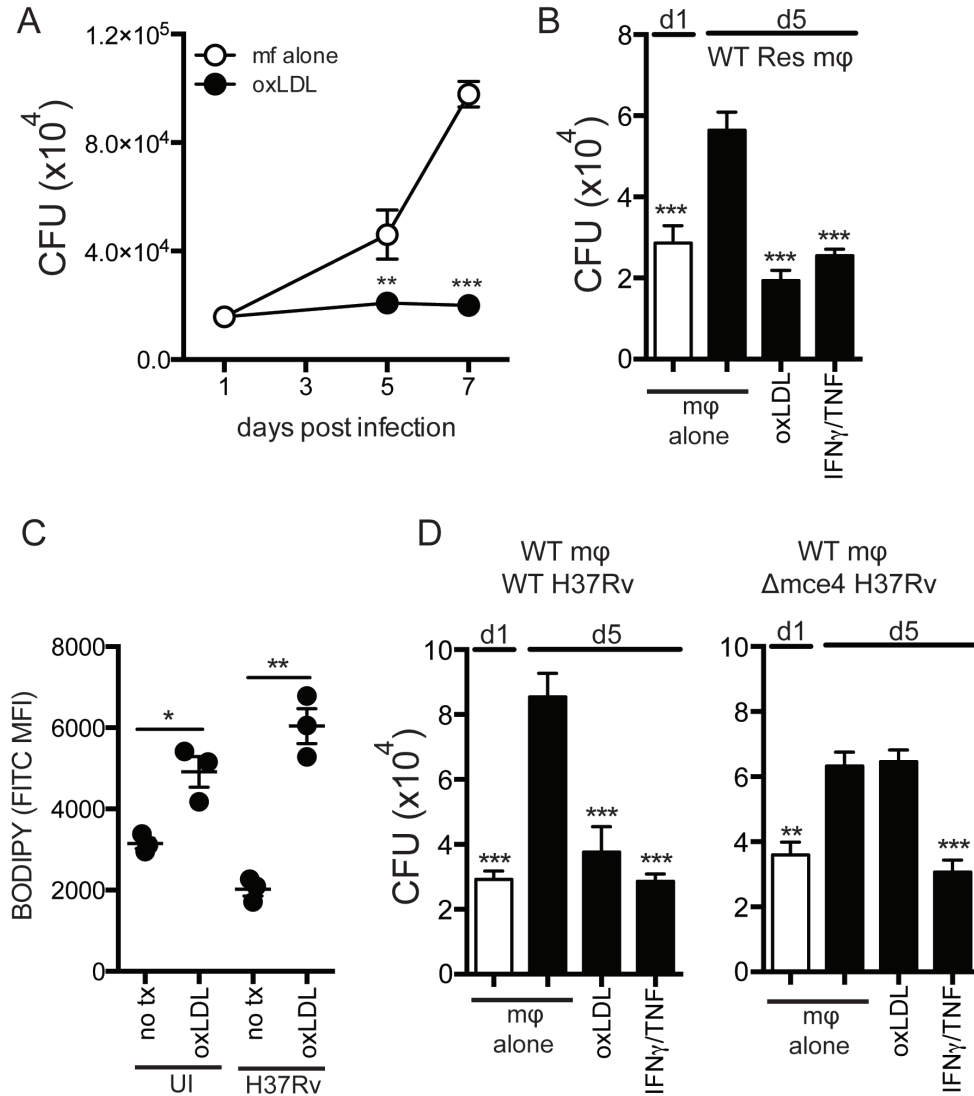


Figure 4-8. oxLDL treatment leading to a foamy macrophage phenotype inhibits Mtb growth.

(A) CFU assay d1, d5 and d7 post-infection with H37Rv-infected WT mφ treated on d1 with oxLDL (50 μ g/ml). (B) CFU assay d1 and d5 post-infection with WT H37Rv-infected WT resident peritoneal mφ treated on d1 with oxLDL (50 μ g/ml). (C) oxLDL (50 μ g/ml) was added to uninfected or H37Rv-infected WT mφ 1 day post-infection. After 4 days, cells were stained with BODIPY 493/503 for 20 minutes at 37°C and then fixed. FITC MFI was determined by flow cytometry. (D) CFU assay d1 and d5 post-infection with WT H37Rv and Δ mce4 H37Rv-infected WT TGL-elicited peritoneal mφ treated on d1 with oxLDL (50 μ g/ml). Recombinant IFN γ (10 U/ml) and TNF (10 ng/ml) used for positive control. Error bars indicate mean \pm SEM. *P < .05, **P < 0.01, ***P < .001. (One-way ANOVA with Dunnett's post-test, compared to d5 or d7 untreated mφ (A, B, D) and unpaired student's t-test (C)). Data is representative of two independent experiments (A, C) or are from single experiments (B, D).

DISCUSSION

Here we have shown that recombinant GM-CSF restricts Mtb growth in both murine thioglycollate-elicited peritoneal macrophages and hMDM. GM-CSF-induced growth inhibition requires PPAR γ signaling in host macrophages. By quantitative mRNA analysis, PPAR γ gene expression and several of its downstream target genes decrease modestly in response to GM-CSF stimulation. GM-CSF leads to increases in neutral lipid content as measured by BODIPY 493/503 staining and by mass spectrometry analysis. These increases are accompanied by qualitative changes in the lipid species. Utilizing an Mtb mutant that is unable to take up cholesterol as well as a chemical inhibitor of one of the enzymes required for propionyl-CoA break-down, we have preliminary data that GM-CSF interferes with baseline Mtb lipid metabolism, inducing intracellular stress and slowing Mtb growth. Lastly, using an independent pathway to increase lipid content in the macrophage, we demonstrate that oxLDL treatment of infected macrophages also leads to a decrease in Mtb growth.

While GM-CSF has been associated with many different macrophage functions that can contribute to an antimicrobial response, reports of its role in lipid metabolism so far have been restricted to pulmonary development. The initial observation that the antimicrobial effect of GM-CSF required PPAR γ expression in macrophages led us to consider whether regulation of lipid metabolism could function as an effector function against this intracellular pathogen.

The concept of “nutritional immunity” as a mechanism of the immune system to limit growth of intracellular pathogens by either sequestering or overloading particular nutrients has gained recent attention. Lipid droplets, more specifically, have also become increasingly appreciated as important sites of host-pathogen interactions and immune pathways^{41, 42} and a source of cell membrane components for structures like autophagosomes⁴³.

In addition, two recent articles have linked PPAR signaling to host metabolic changes that affect the outcome of intracellular bacterial infections. PPAR δ was shown to play a detrimental role in *Salmonella typhimurium* infection where its signaling was associated with increased intracellular glucose availability and increased bacterial growth and persistence⁴⁴. Additionally, an

increase in PPAR γ expression that caused a subsequent increases in intracellular glucose accounted for the increase in susceptibility of alternatively-activated macrophages to intracellular *Brucella abortus* infection⁴⁵.

Previous research in pulmonary development found that GM-CSF stimulates PPAR γ expression. Therefore, it was surprising to find that in our system GM-CSF led a modest decrease in PPAR γ gene expression. However, both thioglycollate-elicited peritoneal macrophages and mature alveolar macrophages are both known for their high PPAR γ expression at baseline, and so perhaps GM-CSF has alternative effects under these conditions.

The concept of decreased PPAR γ expression as a host-derived mechanism for mycobacterial control specifically has support from several previous studies that have demonstrated that Mtb induces PPAR γ signaling during infection and inhibiting PPAR γ expression (through RNAi or antagonists) leads to a decrease in bacterial growth. One study found that BCG induced PPAR γ gene expression in both murine and hMDM in a TLR2-dependent manner³². The increase in PPAR γ expression was associated with an increase in lipid bodies and PGE₂ production and these phenotypes were reproduced with PPAR γ agonists. In a second study, the Mtb lipid mannose-capped lipoarabinomannan (ManLam) was shown to induce PPAR γ expression through mannose receptor binding in hMDM leading to p38-MAPK signaling and IL-8 production³³. siRNA PPAR γ knockdown led to a decrease in H37Rv growth within the first 24 hours of macrophage infection. The third study validated that Mtb lipids upregulated PPAR γ expression and blocking expression through RNAi led to a decrease in H37Rv CFU and an increase in iNOS and pro-inflammatory cytokine expression³¹.

Despite the evidence for the PPAR γ signaling pathway promoting Mtb virulence, there is also evidence that LXR α and LXR β , two downstream targets of PPAR γ , are critical for host immunity. LXR α ^{-/-} and LXR α ^{-/-}LXR β ^{-/-} mice have been shown to be highly susceptible to Mtb infection and both prophylactic and therapeutic LXR agonist treatment lead to improved disease outcome⁴⁶. The absence of LXR signaling was associated with increased foamy macrophage formation and increased neutrophilic inflammation. The discrepancy between these published

results points to how complicated and multifaced these host pathways are. Therefore, interpretation of these various results must be made cautiously.

It is still unclear exactly how host cholesterol status affects immunity. While a cholesterol-rich diet was shown to decrease the time to negative culture in pulmonary tuberculosis⁴⁷, it has also been shown that ApoE^{-/-} mice on a high-fat diet with severe hypercholesterolemia have higher susceptibility to Mtb infection due to a delayed adaptive response and a decreased pro-inflammatory response⁴⁸.

These studies clearly show that changes in host lipid metabolism may have a wide range of influences during infection, at the level of the macrophage, the priming of the adaptive immune response, as well as more systemic inflammation and cellular recruitment. Much more research will need to be done to tease these different effects apart. It is also likely that for host lipid metabolism to be effective at altering the growth of pathogens, the host must hit upon a perfect equilibrium, where modest amounts of cholesterol contribute to bacterial control but too much leads to an inflammatory environment and immunopathology. Perhaps an endogenous signal like GM-CSF could achieve these types of modest and effective metabolic changes.

For chronic infections, it is an appealing model that the immune system could alter the pathogen's environmental niche to slow its growth and allow other microbicidal mechanisms time to act. Specifically for tuberculosis, these types of host-directed therapy targets could be incredibly useful, especially in the context of drug resistance.

Ultimately, understanding the metabolic vulnerability of Mtb in the context of a host macrophage could reveal novel therapeutic targets that would not be seen in the absence of the host response. An example of how this strategy could be effective was recently performed by testing for growth of mycobacterial mutants in the presence and absence of a CD4⁺ T cell immune response through a transposon library screen (TraSH) (*see Appendix II*). This screen identified an Mtb tryptophan production pathway that was only required in the presence of CD4⁺ T cells and was shown to synergize with IFN γ -mediated IDO production that depleted host tryptophan availability⁴⁹. This study is one example of how the immune response alters the environmental

niche for Mtb, resulting in new nutritional vulnerabilities for the pathogen that can be taken advantage of therapeutically.

Overall, this study demonstrated that the antimicrobial mechanism of GM-CSF requires host PPAR γ -signaling and GM-CSF likely generates metabolic stress for the intracellular bacteria by altering host cellular metabolism. These results help to both characterize the antimicrobial function of GM-CSF as well as elucidate new mycobacterial pathways to target during Mtb infection.

MATERIALS AND METHODS

Ethics Statement

All mice were bred and maintained using standard humane animal husbandry protocols. All animal experiments were performed in accordance with relevant guidelines for the care and handling of laboratory animals and were approved by the Dana Farber Cancer Institute Animal Care and Use Committee (Animal Welfare Assurance number A3023-01) or by the Department of Animal Medicine of the University of Massachusetts Animal Care and Use Committee (Animal Welfare Assurance number A2420-13) under Public Health Service assurance of the Office of Laboratory Animal Welfare guidelines.

Mice

C57BL/6J (WT) mice were obtained from Jackson Laboratories. C57BL/6J PPAR $\gamma^{fl/fl}$;LysMcre $^{+/+}$ (PPAR $\gamma^{-/-}$) and PPAR $\gamma^{fl/fl}$; LysMcre $^{-/-}$ (PPAR $\gamma^{fl/fl}$ control) were provided by Dr. Glenn Dranoff.

Macrophage isolation and culture

Thioglycollate (TGL)-elicited peritoneal macrophages were lavaged 4–5 days after 3% intraperitoneal TGL injection and then isolated by positive selection with CD11b microbeads and LS magnetic columns (Miltenyi Biotec). Purified cells were over 95% F4/80 $^{+}$ CD11b $^{+}$, as determined by flow cytometry. Bone marrow-derived macrophages (BMDM) were differentiated from bone marrow after 7 days in RPMI supplemented with 20% L929 cell supernatant. Primary human peripheral blood (Research Blood Components, Boston, MA) monocyte-derived macrophages (MDM) were generated from adherent bead-selected CD14 $^{+}$ cells after 7 days in culture with human serum (Gemini Bioproducts). Macrophages were seeded at 1×10^5 in 96-well culture plates in complete RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% fetal calf serum (HyClone).

Mtb in vitro culture and infection

H37Rv was grown and prepared as previously described⁵⁰. Bacteria was counted and added to macrophages at an effective multiplicity of infection (MOI) of 0.2 for CFU experiments for two hours. Cultures were washed three times to remove extracellular bacteria. Infected macrophages were cultured overnight and iNKT cells or other conditions were added on d1. For CFU measurement, cells were lysed with 1% Triton X-100/PBS and lysate from quadruplicate conditions were plated in serial dilutions on Middlebrook 7H10 agar plates (ThermoFisher Scientific), and cultured at 37°C for 21 days. Infected macrophages were treated with the following reagents: recombinant GM-CSF (murine, Peprotech; human, Biolegend), recombinant IFN γ (murine, Peprotech), 3-nitropropionate (Sigma-Aldrich), vitamin B12 (Sigma-Aldrich), and oxLDL (Biomedical Technologies). The Δ mce4 H37Rv mutant strain was provided by Dr. Chris Sassetti³⁵.

Quantitative RT-PCR

RNA was isolated from macrophages with Trizol and Purelink RNA Mini Kit (Invitrogen) according to the manufacturer's instructions. cDNA was prepared using the SuperScript RNA amplification system (Invitrogen) according to the manufacturer's instructions. Quantitative PCR assays were performed using Power SYBR Green and Fast SYBR Green (Invitrogen) systems on a ViiA 7 QPCR machine (Applied Bioscience). Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as reference gene. Specific oligonucleotides were used for *Hprt* (sense, 5'-GCT GGT GAA AAG GAC CTC T-3', antisense, 5'-CAC AGG ACT AGA ACA CCT GC-3'). Primers for *Pparg*, *Lxra*, *Lxrb*, *Apoe*, and *iNOS* were used as published by Korf et al⁴⁶. For the Fast SYBR Green assays (60°C melting temperature only), the cDNA was denatured for 1 min at 95°C, followed by 40 cycles of 95°C for 3 sec, annealing and extending at 60°C for 30 sec. For the Power SYBR Green assays (all other melting temperatures), the cDNA was denatured for 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, incubation at the optimized melting temperature for 30 sec, and 72°C for 30 sec. Optimized melting temperatures were 60°C for *Hprt*, *Lxrb*, 55°C

for *Pparg*, *iNOS*, and 53°C for *Apoe*, *Lxra*. The expression level of each gene was determined using the $\Delta\Delta\text{Ct}$ method. Data are represented as the ratio of the expression level of the gene for each stimulation condition over the mean expression level of the gene in uninfected untreated macrophages.

BODIPY Neutral Lipid Staining

Adherent macrophages were washed once with incomplete RPMI and then incubated for 20 minutes with 10 μM BODIPY in complete RPMI at 37°C. Macrophages were then washed once in FACS buffer (1% FBS and 0.01% sodium azide in PBS) and then fixed with PFA (1% for flow cytometry and 3.7% for microscopy) for 45 minutes. For flow cytometry, macrophages were then washed in FACS buffer and scraped off the plate and analyzed with the MacsQuant flow cytometer (Miltényi). For microscopy, macrophages were stained with Alexa Fluor® 568 Phalloidin (Invitrogen) staining F-actin, before affixing to microscopy slides with Prolong Gold plus DAPI (Invitrogen). Images were taken with a Leica DM IRE2 Fluorescence and Phase Contrast Microscope at 63X.

Liquid Chromatography-Mass Spectrometry

Macrophages were seeded at 1×10^6 per well in 24-well plates and untreated or stimulated for 4 days with GM-CSF (10 ng/ml). They were then washed with PBS, scraped, and pelleted. After the remaining liquid was removed, the pellets were flash frozen in dry ice and stored at -80°C. The Folch extraction method⁵¹ in combination with NH2 Bond Elute SPE 50mg cartridge (Agilent) was used to extract neutral lipids from the cell pellets. Neutral lipid standards were obtained from Avanti Lipids (Alabaster, Alabama), 1-heptadecanoyl-rac-glycerol (17:0 MG), 1,2-dilauroyl-*sn*-glycerol (12:0/12:0 DG), 1,3(d5)-ditetradecanoyl-2-(9Z-hexadecenoyl)-glycerol (14:0/16:1/14:0 TG-D5), cholest-5-en-3 β -yl pentadecanoate (15:0 cholesterol ester), 1-oleoyl-N-heptadecanoyl-D-*erythro*-sphingosine (17:0/d18:1 ceramide). Cholesterol-D7 was obtained from Cambridge Isotope Labs. The neutral lipid standards were dissolved in methanol at a concentration of 1mg/mL to

keep as stock solution. They were then mixed to 0.2 ng/μl of TG/MG/cholesteryl Ester, 0.1 ng/μl of DG, 2 ng/μl of ceramide, and 20 ng/μl of cholesterol.

The neutral lipid fraction was resuspended in 100 μl of mobile phase A and mixed with 100 μl of the neutral lipid standard mix. 20 μl of each sample was injected on to a Kinetex 2.6u C18 100A 150 x 2.1 mm column (Phenomenex) using an Accela (Thermo Fisher Scientific) auto sampler and Accela 1250 LC-pump and analyzed in triplicate. Elution of the C18 column was carried out with (A) 95% Methanol, 5% 100 mM Ammonium Formate, pH 4.75 and (B) 95% Isopropanol, 5% 100 mM ammonium formate, pH 4.75. The flow rate was at 150 μl/min and the gradient was as follows: 0 min 50% B, 5 min 50% B, 20 min 100% B, 30 min 100% B, 30.1 min 100% B with a 15 min re-equilibration time at the end of the run. Full scan MS spectra were obtained using positive ion electrospray MS using a LTQ Orbitrap Velos (Thermo Scientific) at 60000 resolution with data dependent MS² scans of the top 5 most intense ions in the MS spectra.

% CFU reduction

To compare inhibition of bacterial growth across multiple experiments, % CFU reduction was calculated. 100% CFU reduction on d5 indicates complete inhibition of bacterial growth to d1 levels while 0% CFU reduction indicates no change in bacterial growth from untreated macrophages. $\% \text{ CFU reduction} = 100 \times [CFU_{(\text{untreated mf-d5})} - CFU_{(\text{treated mf-d5})}] / [(CFU_{(\text{untreated mf-d5})} - CFU_{(\text{untreated mf-d1})})]$

Statistical analysis

All data are represented as mean ± SEM. Data was analyzed by One-Way ANOVA (95% confidence interval) with Dunnett's post-test (for comparison against a single control) or unpaired (multiple) Student's t-test. Analysis was performed using GraphPad Prism software.

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Chapter 5: Discussion

Despite improved access to effective antibiotics and increased investment in medical infrastructure, the TB epidemic continues to be a global health crisis. The emergence of multi-drug resistant and extensively-drug resistant strains necessitates the continual search for novel therapeutic targets and pathways.

The last two decades of research have identified many immune pathways involved in protection against Mtb infection. Although basic research has characterized a variety of mechanisms, vaccine work has primarily focused on increasing the number and frequency of antigen-specific CD4⁺ T cells and their production of IFN γ . The absence of these factors dramatically increases the susceptibility of mice as well as people to Mtb infection^{1, 2, 3, 4}. However, an increase in these measurements has not been associated with immune protection. In fact, it is often correlated with progression to active disease and bacterial burden^{5, 6, 7, 8}. In light of the recent failure of the MVA85A vaccine Phase IIb clinical trial, where MVA85A did not show increased protection over standard BCG treatment despite generating enhanced CD4⁺ T cell IFN γ and TNF responses, it is critical to reassess this model^{9, 10}.

The work in this thesis focused on characterizing new antimicrobial roles for previously known immune components: iNKT cells and GM-CSF. These studies tried to ignore assumptions about known pathways (i.e. IFN γ production by iNKT cells) and test the requirement for these different effector functions in an in vitro macrophage infection model. This research revealed a novel iNKT cell effector function and new roles for GM-CSF in restriction of bacterial growth during Mtb infection. In addition, the study of these pathways produced mechanistic insight into the host-pathogen interface during Mtb infection.

SUMMARY OF FINDINGS

The research in this thesis focused on antimicrobial effector functions during Mtb infection. **Chapter 2** focused on iNKT cell activation and effector function during Mtb infection. The impetus for this work came from two previous observations about iNKT cells and Mtb. The first observation was an in vivo study that treatment of both C3H/HeJ and BALB/c mice with

α GalCer led to increased survival and decreased bacterial burden after aerosol infection in a CD1d-dependent manner¹¹. The second was an in vitro study that addition of naïve iNKT cells to H37Rv-infected macrophages led to a restriction in bacterial growth¹².

The aim of this study was to understand how iNKT cells were protective during infection and which activation pathways were required. Here, we showed that iNKT cells became activated following co-culture with Mtb-infected macrophages. IL-12 and IL-18 cytokine signaling induced expression of activation markers and IFN γ production by iNKT cells. However, these signals did not contribute to iNKT cell antimicrobial activity. In contrast, CD1d-driven activation was critical for the ability of iNKT cells to inhibit Mtb growth but had little effect on other markers of activation. Using IFN γ ^{-/-} iNKT cells and IFN γ R^{-/-} macrophages, we were able to show that iNKT cells control Mtb independently of IFN γ . We next looked for an IFN γ -independent effector function that was stimulated after CD1d signaling. We identified GM-CSF as a cytokine produced by iNKT cells in response to Mtb-infected macrophages in a CD1d-dependent manner. Blocking GM-CSF inhibited the antimicrobial effect of iNKT cells and recombinant GM-CSF was sufficient to restrict Mtb growth. We could detect GM-CSF-producing iNKT cells in the lungs of Mtb-infected mice early during infection and this production also appeared to be CD1d-dependent.

In **Chapter 3**, we examined a role for T cell-derived GM-CSF during Mtb infection. By Bioplex immunoassay, we could detect an increase in GM-CSF protein in the lung over the course of infection. By ICS analysis, GM-CSF production was detected for 4 different T cell subsets: iNKT cells, $\gamma\delta$ T cells, CD4⁺ and CD8⁺ T cells. Each T cell subset displayed different kinetics of GM-CSF production over the course of 8-12 weeks of infection. The dynamics of GM-CSF production differed from the patterns of expression of IFN γ production, suggesting that the regulation of these two cytokines were independent. Next, using an adoptive transfer model, we tested the contribution of T cell-derived GM-CSF to control of Mtb. Preliminary evidence showed that Rag^{-/-} mice that received GM-CSF^{-/-} T cells had approximately a half log increase in bacterial burden by week 4 compared to Rag^{-/-} mice that received WT T cells. This data suggested that

even in the presence of other cytokine and effector functions, GM-CSF has a non-redundant role in reducing bacterial growth during Mtb aerosol infection.

Chapter 4 followed up on these results by investigating the direct antimicrobial mechanism of GM-CSF on infected macrophages. We found that the effect of GM-CSF required host macrophage PPAR γ -signaling and led to a modest decrease in PPAR γ gene expression. GM-CSF increased the total neutral lipid content of macrophages, as measured by BODIPY 493/503 staining and this was independent of PPAR γ -signaling. Next, we examined the identity of the neutral lipid species within the treated macrophages and found significant changes in response to GM-CSF stimulation including increases in TAG, decreases in ceramides, and changes in cholesterol esters and free cholesterol species that may potentially be regulated by PPAR γ . To assess how these host metabolic changes may alter the host-pathogen interface, we utilized an Mtb mutant strain, $\Delta mce4$ that lacks cholesterol import machinery. GM-CSF was unable to inhibit growth of the $\Delta mce4$ strain, suggesting that cholesterol uptake is required for the effect of GM-CSF. In addition, we used a chemical inhibitor that blocks one of the enzymes involved in cholesterol breakdown to provide additional evidence that GM-CSF may be inducing host metabolic changes that triggers bacterial metabolic stress, which in turn impedes bacterial growth. To address whether an increase in host lipids by another means would also impede Mtb growth, we treated macrophages with oxLDL and again measured inhibition of growth. These data support a model in which GM-CSF increases macrophage lipid content in a PPAR γ -dependent manner to disrupt the metabolic equilibrium of the host phagosomes and inhibit Mtb growth.

IMPLICATIONS

iNKT cell effector functions

iNKT cells have a unique role in the immune system as T cells that have acquired early innate function and the ability to rapidly respond following primary stimulation. With expression of a semi-invariant TCR, iNKT cells are found at a fairly high frequency in most organs, when

considered as a clonal population rather than an entire T cell subset. As described in detail in *Chapter 1* in *Tables 1-2, 1-3, and 1-4*, a role for iNKT cells has been found in numerous bacterial, viral, and fungal infections. However, the effector functions they utilize to achieve this are untested in many cases. iNKT cells have been coined the “Swiss army knife” of the immune system¹³ because of the multitude of effector functions they are capable of performing (see *Figure 1-3*), so it is surprising that so few of these functions have been tested in the context of infection. The finding that iNKT cell production of GM-CSF controls Mtb infection is likely just one example among many of iNKT cell effector functions that are independent of IFN γ . The key to identifying these functions is the ability to isolate the conditions under which iNKT cells are tested. The difficulty with using whole mouse studies to address mechanism is that in vivo iNKT cells probably have multiple functions and transactivate many cell types, which could be hard to dissociate at the whole organism level. While in vitro studies suffer from synthetic conditions, they can offer the perfect opportunity to isolate specific iNKT cell functions and test them under rigorous conditions.

In addition, our studies highlighted the strong influence of initial activation signals on subsequent effector functions. While both cytokine and CD1d-signaling activated iNKT cells in the context of Mtb infection, they induced very distinct effector programs. This finding predicts that different stimuli from different pathogens will induce distinct iNKT cells effector functions and that these effector functions may even change over the course of the infection as the dynamics of various activation signals change. These ideas hint at a wealth of new mechanisms to discover and high-throughput technologies like the Immgen microarray platform may further expand the opportunities to study them¹⁴.

iNKT cells and Mtb Infection

Ever since mice lacking iNKT cells were shown to be equally resistant to Mtb infection as WT mice^{15, 16, 17}, motivation to study a role for iNKT cells during Mtb infection has been restrained. Interestingly, the more persuasive evidence to study iNKT cells during Mtb infection comes from

human studies. Four independent studies found that iNKT cell frequency and number are decreased in the peripheral blood of patients with active TB compared to either household contacts, patients on treatment, or healthy controls^{18, 19, 20, 21}. In addition, two studies found a change in iNKT cell subset frequency in TB patients, with a decrease in CD4⁻CD8⁻ (DN) iNKT cells and an increase in CD4⁺ iNKT cell subset in the periphery^{20, 22}. Clearly, Mtb infection alters iNKT cell trafficking, but how should these observations be interpreted in the context of iNKT cell biology? Is this evidence that iNKT cells are recruited to the lung, the site of infection, during disease? Does this suggest that iNKT cells are activated and then deleted? Or, does this indicate that iNKT cells are simply “diluted out” by the proliferation of other immune cell subsets responding to infection? Only more thorough clinical studies can address these types of questions.

It is possible that the murine model of Mtb is not representative of human disease when it comes to nonconventional T cell activity. Interestingly, other non-conventional T cells including group I CD1-restricted T cells, MAIT cells, and $\gamma\delta$ T cells have all been shown to respond to human Mtb-infected macrophages and are found in people during infection, but none of them have been shown to be required in mice (note: mice do not possess group I CD1-restricted T cells)^{1, 23, 24, 25, 26, 27}. In addition, stimulation of $\gamma\delta$ T cells was recently shown to enhance resistance of non-human primates during Mtb infection²⁸. Due to the dynamics of chronic infection in mice, where antigen burden remains very high over the course of infection, it is possible that the murine model is not the right one to assess function of these cell types during infection.

Despite lack of evidence that iNKT cells are required for infection in the mouse model, studies from our group have found that activation of iNKT cells with α GalCer leads to increased survival of susceptible mouse strains¹¹ and works in conjunction with antibiotics to improve disease outcome²⁹. In addition, iNKT cells are able to inhibit Mtb growth when added during in vitro co-cultures and the work in this thesis has now identified that iNKT cells produce GM-CSF in response to infection and this is protective¹².

How can these data be reconciled? It is clear that iNKT cells, once activated, are protective during infection. Then why are they not required for bacterial control in the murine model? There are at least two possible explanations. First, their function is redundant with other cell types. Work in *Chapter 3* revealed that iNKT cells are not the only T cell or innate subset to produce GM-CSF in the lung during Mtb infection. If GM-CSF is the dominant iNKT cell effector function, it is easy to imagine how GM-CSF coming from other sources would make iNKT cells redundant.

Second, the chronic nature of Mtb may make it a poor fit for iNKT cell-mediated control. Many of the infections for which iNKT cells are required for pathogen control (*Tables 1-2, 1-3, and 1-4*) are acute infections that are resolved within several days to a week. Because protection against Mtb infection requires weeks of function, iNKT cells may participate very early on during infection, but become less functional over long periods of activation. A previous study with BCG infection found that iNKT cells become anergic over time³⁰. After 7 days of infection, iNKT cells started to die, stopped producing cytokines, and became unresponsive to further stimulation, just as conventional T cells were starting to proliferate and respond. This timing suggests that iNKT cells might serve as a bridge between the innate and adaptive immune system instead of being central mediators of control. Further investigation into these immune dynamics should be performed.

Antimicrobial role for T cell-derived GM-CSF

GM-CSF has long been considered to be a myeloid and granulocyte growth factor. It is used at the clinical bedside to rescue chemotherapy-induced neutropenia and at the bench to generate bone marrow-derived dendritic cells^{31, 32}. GM-CSF has also recently been recognized as a factor driving Th17-induced autoimmune disorders^{33, 34} and antagonists are now in clinical development to treat rheumatoid arthritis and multiple sclerosis³².

The identification of T cell-derived GM-CSF as a direct antibacterial factor adds a new function to the T cell antimicrobial arsenal and highlights an additional role for GM-CSF. For

tuberculosis specifically, recombinant GM-CSF (rGM-CSF) has recently been used successfully in murine treatment studies^{35, 36}. Only a single human trial has ever tested rGM-CSF during active TB³⁷. Although it was well tolerated, it failed on the endpoints tested. Because few aspects of disease were characterized during this trial, it is unclear why it failed. However, an evident misstep was the route of administration. In this trial, rGM-CSF was given subcutaneously, but previous research found that aerosol but not systemic delivery of GM-CSF restores pulmonary function in GM-CSF^{-/-} mice³⁸. It appears that the route of delivery is critical for attaining biological effects with rGM-CSF in mice and whether this is also true in people has not yet been tested.

While rGM-CSF has many appealing attributes for a potential immunotherapy during tuberculosis, the contribution of GM-CSF to autoimmune conditions necessitates caution in reviewing the details of how and for how long it would be administered. Like most factors in biology, striking an equilibrium between too little and too much GM-CSF is likely to be most beneficial for treatments.

Due to the short half-life of rGM-CSF and the presumed need to fine-tune the dosage of the cytokine in vivo, investigating means to induce T cell production of GM-CSF production endogenously may be more effective therapeutically. Because so many T cell subsets are capable of producing GM-CSF, this leaves many different therapeutic routes available for future investigation.

Effect of lipid content on macrophage antimicrobial activity

Because Mtb is an intracellular and chronic pathogen, it works very hard to maintain an intracellular niche that allows for persistence, growth, and ultimately replication. It blocks phagosome maturation and vesicular acidification^{39, 40}. Mtb utilizes the glyoxylate shunt and gluconeogenesis pathways to access carbon sources^{41, 42}. It also expresses sensors like DosRS and enzymes like catalase-peroxidase to perceive and repair oxidative stress^{43, 44}. In addition, Mtb requires amino acid biosynthesis and/or access to host amino acids for growth⁴⁵.

Therefore, it is not surprising that immune cytokines might alter host cell metabolism in an effort to make these intracellular niches less hospitable. For example, a recent study found both IFN γ and LPS stimulation induced host immunoresponsive gene 1 (*Irg1*) in macrophages that generated a host metabolite itaconic acid^{46, 47}. Itaconic acid acted as an isocitrate lyase inhibitor, blocking glyoxylate shunt activity and restricting bacterial growth in macrophages. This demonstrates that host inflammatory signals can lead to bacterial metabolic pressure that acts as an antimicrobial mechanism.

The results that GM-CSF leads to an increase in neutral lipid content and an increase in cholesterol in combination with studies utilizing Mtb metabolic mutants and chemical inhibitors suggest that these pathways may be critical to the antimicrobial function of GM-CSF. The model that increased cholesterol quantity within the host cell is detrimental to Mtb growth may at first appear counterintuitive. It has been demonstrated that Mtb can utilize cholesterol as a carbon source and its growth in cholesterol is normal⁴⁸. It would appear that increased access to this carbon source would allow Mtb to grow even better. However, it has been documented that Mtb is highly vulnerable to effects of cholesterol toxicity due to accumulation of propionyl-CoA and other metabolites toxic to the cell^{49, 50, 51}. These effects can be rescued by either increased propionyl-CoA catabolism or increased acetyl-CoA levels. In addition, one hypothesis is that changing the access to carbon sources, even if they are increased, may alter Mtb cell wall permeability and integrity, leading to inhibited growth.

Results of in vivo experiments are much harder to interpret in the context of these pathways. For example, a recent study demonstrated that treatment of Mtb-infected mice with statins, HMG-CoA reductase inhibitors that lead to lowering of cholesterol levels, were effective in reducing bacterial burden in mice⁵². In addition, hMDM from patients on statin therapy showed increased resistance to Mtb growth during in vitro cultures. However, this result must be interpreted carefully, because statins have been shown to decrease cholesterol levels in the blood leading to subsequent decreased cholesterol efflux from macrophages and potential contradictory accumulation of cholesterol in peripheral cells⁵³. It is not clear yet whether the

beneficial effect of statins during Mtb infection comes from reduced cholesterol within host cells or whether other systemic changes account for the effect.

Whether GM-CSF could ever be used therapeutically or induced immunologically to control infection remains to be seen. However, examining the metabolic consequences of GM-CSF both on the host and bacterial sides may reveal other novel and more feasible therapeutic targets. For example, both PPAR γ and LXR have well-developed agonists and antagonists that could be adapted for therapeutic use. Investigation into the antimicrobial role of GM-CSF will hopefully provide greater insight into the biology behind the macrophage-Mtb interaction.

FUTURE DIRECTIONS

There are many potential paths of further investigation into the antimicrobial roles for both iNKT cells and GM-CSF. In order to better understand the role for iNKT cell activation during Mtb infection and link various observations that have been made, it would be important to investigate the effector function utilized by iNKT cells to reduce bacterial growth after in vivo α GalCer stimulation. Given our in vitro co-culture results using IFN γ ^{-/-} iNKT cells and Mtb-infected macrophages, we would predict that iNKT cells do not require IFN γ production for this effect and in fact produce GM-CSF as the dominant effector function. However, this experimental set-up is complicated by the fact that iNKT cells from GM-CSF^{-/-} mice are dysfunctional, likely due to the requirement of GM-CSF in the maturation of the cells selecting iNKT cells during development⁵⁴. Therefore, to test this hypothesis, a more complicated transfer experiment will need to be performed. In addition, it would be useful to investigate whether iNKT cells can be activated therapeutically in people to induce GM-CSF production and restrict bacterial growth.

We demonstrated that many of the T cells that produce GM-CSF during infection also produce IFN γ . To fully understand the effect of GM-CSF during infection, studies investigating the function of GM-CSF in the presence of other cytokines should be undertaken. We have preliminary evidence that GM-CSF and IFN γ have additive protective effects in vitro. In addition, there are reports that GM-CSF induces the production of pro-inflammatory cytokines like TNF and

IL-1 β . New evidence that IFN γ signaling in macrophages induces itaconic acid that acts as an isocitrate lyase inhibitor also provides preliminary evidence that the protective effects of IFN γ and GM-CSF may be linked mechanistically^{46, 47}.

Utilizing the $\Delta mce4$ Mtb mutant was very informative for testing how GM-CSF stimulation affected Mtb growth. There are several other known metabolic mutants that could also be useful to test, but a more thorough analysis of bacterial pathways that are affected by this stimulation could be accomplished by screening with a transposon mutant library⁵⁵. This could be accomplished either in GM-CSF treated macrophages or in GM-CSF^{-/-} mice compared to appropriate controls and provide further insight into both the antimicrobial effects of GM-CSF and the transcriptional program utilized by Mtb to counteract host immune stresses.

Another area of future investigation is the effect of GM-CSF on human macrophages. In *Chapter 4*, we tested rGM-CSF on hMDM and observed greater heterogeneity in the response of human macrophages to GM-CSF than in murine cells. Previous studies that described the protective effect of GM-CSF in hMDM did not comment on donor-to-donor variability^{56, 57, 58}. It is not yet clear whether this variability is technical or biological. If inducing lipid changes in the host macrophage generates the antimicrobial effect of GM-CSF, then one would predict that the host lipid status at baseline would influence the magnitude of the effect of GM-CSF. This is a consideration at the cellular level and also at the host level. The interaction between host metabolism and tuberculosis has gained greater attention in light of the increased susceptibility to TB by people with diabetes mellitus and the current epidemiological shift that is bringing these two public health epidemics closer together⁵⁹. In considering future therapeutic approaches using GM-CSF, variability in the host response due to conditions of lipid dysregulation, like diabetes, will certainly need to be addressed.

Lipid droplets have been described during infection as potential sites of both nutrient acquisition by pathogens and microbial killing^{60, 61}. Lipid droplets can associate with phagosomes and transport of lipid content between these organelles has also been observed. It has been hypothesized that lipid droplets could play a role in accelerating phagolysosomal fusion as well as

enhancing autophagy pathways⁶². One hypothesis is that the increase in neutral lipids observed after GM-CSF treatment may induce increases in phagosome maturation and autophagy that contribute to the inhibition in Mtb growth. The effect of increased lipid content, especially stored in lipid droplets, on other cellular processes could be investigated further.

Roles for both iNKT cells and GM-CSF during Mtb infection offer many new avenues for investigation. A better understanding of both factors will offer greater mechanistic insight into TB immunity. This study is just the first step towards addressing these new mechanisms.

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Appendices

I. Supplemental Figures: GM-CSF antimicrobial mechanism

II. Additional Publications

Experimental assistance from Kassie Dantzler and Pushpa Jayaraman.

APPENDIX I: SUPPLEMENTAL FIGURES

GM-CSF antimicrobial mechanism

To determine how GM-CSF leads to restriction of bacterial growth in mature macrophages, a number of common effector functions and macrophage characteristics were analyzed. These included nitric oxide (NO) production, reactive oxygen species (ROS) production, phagocytosis rates, cell survival and growth, and cell cycling. Interestingly, GM-CSF appeared to have little effect on most of these measurements, with the exception of increased cell growth and cell cycling. This is likely because many of the reports of GM-CSF inducing these phenotypes may have in fact been measuring the maturation effect of GM-CSF on immature monocytes, rather than the effect of GM-CSF on fully mature activated macrophages, as observed with our thioglycollate-elicited peritoneal macrophages.

The following results are included here:

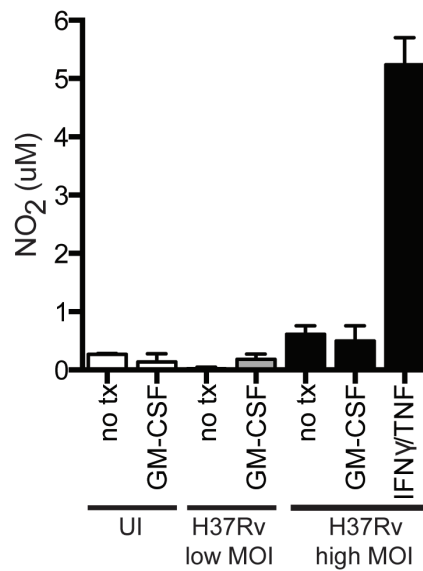
Supplemental Figure 1. Recombinant GM-CSF does not induce nitric oxide production.

Supplemental Figure 2. Recombinant GM-CSF does not induce ROS production.

Supplemental Figure 3. Recombinant GM-CSF increases CD80 and MHC II expression but does not increase the rate of phagocytosis.

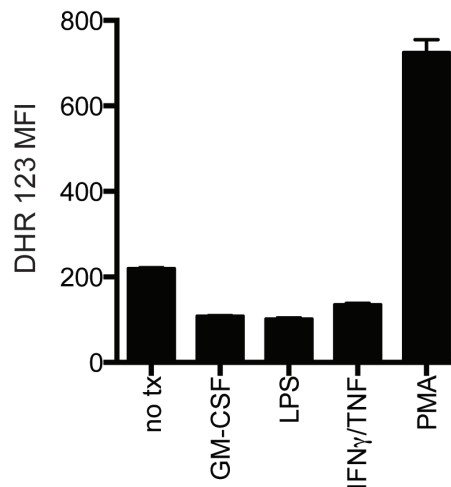
Supplemental Figure 4. Recombinant GM-CSF increases cell survival and growth.

Supplemental Figure 5. Recombinant GM-CSF alters macrophage cell cycling.



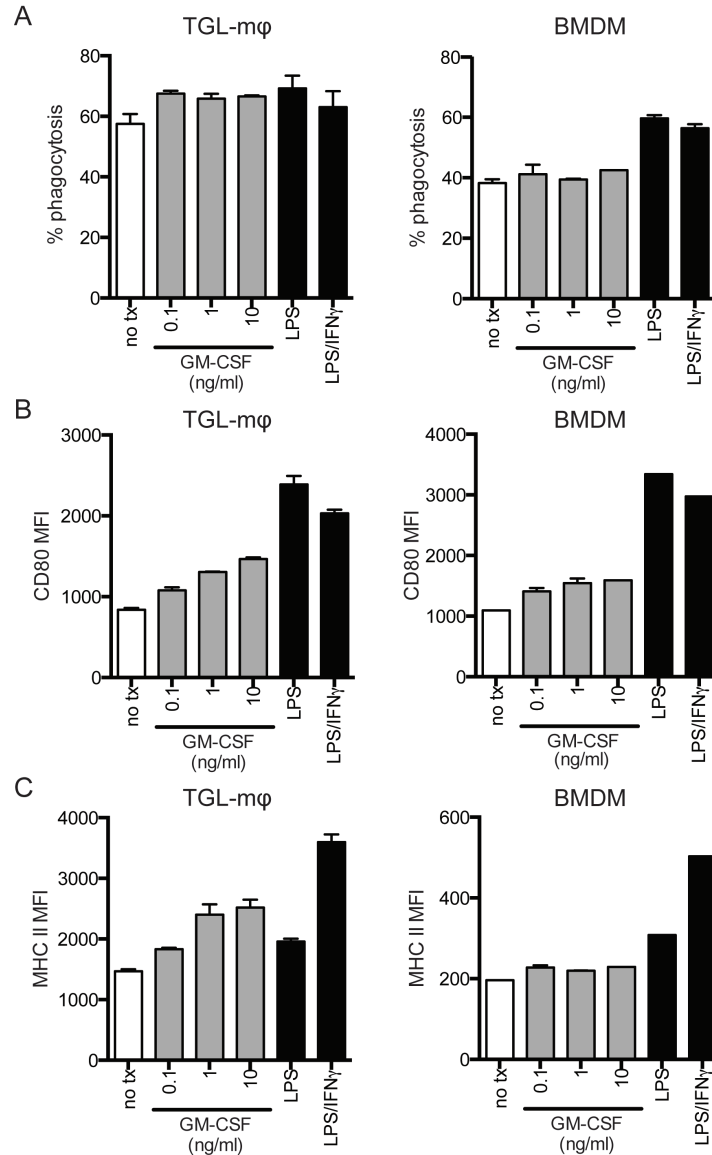
Supplemental Figure 1. Recombinant GM-CSF does not induce nitric oxide production.

Uninfected or H37Rv-infected WT murine mφ treated after 24 hours with GM-CSF (10 ng/ml) or IFNγ (10 U/ml) and TNF (10 ng/ml). Supernatant was harvested at 48 hours post-infection, 24 hours post-treatment. Nitrite, a stable breakdown product of NO₂, was measured by Griess reaction. Error bars indicate mean ± SEM. Data is from triplicate samples from one experiment.



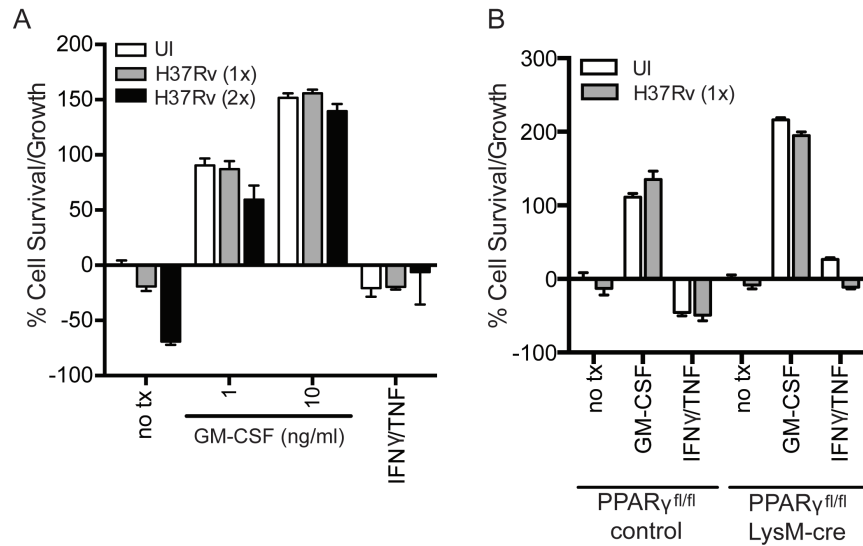
Supplemental Figure 2. Recombinant GM-CSF does not induce ROS production.

Uninfected WT murine mφ treated for 24 hours with GM-CSF (10 ng/ml), LPS (5 ng/ml), IFNγ (10 U/ml) and TNF (10 ng/ml), or PMA as a positive control. Cells were then stained with dihydrorhodamine (DHR) 123 and fluorescence was measured by flow cytometry. Error bars indicate mean ± SEM. Data is from triplicate samples from one experiment.



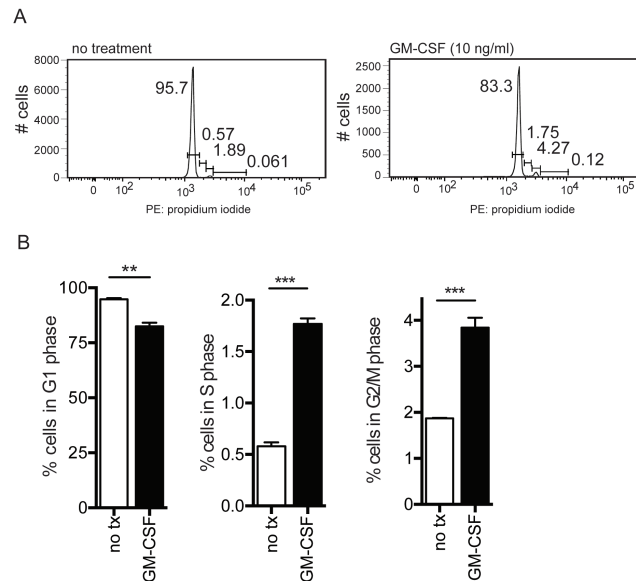
Supplemental Figure 3. Recombinant GM-CSF increases CD80 and MHC II expression but does not increase the rate of phagocytosis.

Uninfected WT TGL-elicited peritoneal mφ (TGL-mφ) and bone marrow derived macrophages (BMDM) treated for 24 hours with GM-CSF (0.1-10 ng/ml), LPS (1 ug/ml), or LPS/IFNγ (1 ug/ml; 10 U/ml) as positive controls. (A) % phagocytosis measured after treated mφ were fed fluorescently tagged heat-killed *E. coli* for 30 mins (B and C). CD80 and MHC II surface expression measured by flow cytometry. Error bars indicate mean ± SEM. Data is from duplicate samples from one experiment.



Supplemental Figure 4. Recombinant GM-CSF increases cell survival and growth.

WT TGL-elicited peritoneal mφ (TGL-mφ) (A) and PPAR $\gamma^{fl/fl}$; LysM-cre mφ and PPAR $\gamma^{fl/fl}$ control mφ (B) were either uninfected or infected with H37Rv at varying MOIs and treated on d1 with GM-CSF (1-10 ng/ml) or IFN γ (10 U/ml) and TNF (10 ng/ml). Cell survival and growth was measured on d5 using the crystal violet assay. Error bars indicate mean \pm SEM. Data is representative of two experiments (A) or from a single experiment (B).



Supplemental Figure 5. Recombinant GM-CSF alters macrophage cell cycling.

WT TGL-elicited peritoneal mφ were treated for 48 hours with GM-CSF (10 ng/ml) and then stained with propidium iodide. DNA content was measured by flow cytometry and various DNA peaks were isolated to determine cell cycle phase. (A) Representative flow cytometry histograms for propidium iodide staining. (B) % of the macrophage population in G1, S, or G2/M phase. Error bars indicate mean. Data is representative of a single experiment.

APPENDIX II: ADDITIONAL PUBLICATIONS

1. *EspA acts as a critical mediator of ESX1-dependent virulence in Mycobacterium tuberculosis by affecting bacterial cell wall integrity.*

Garces A, Atmakuri K, Chase MR, Woodworth JS, Krastins B, **Rothchild AC**, Ramsdell TL, Lopez MF, Behar SM, Sarracino DA, Fortune SM. *PLoS Pathog.* 2010 Jun 24;6(6) e1000957.

The ESX-1 secretion system in Mtb is highly associated with its virulence. However, the effects of individual secreted factors by ESX-1 had not been studied separately from the total effect of the secretion system, because deletion of any individual component completely inhibited all secretion. This study sought to dissociate these virulence factors by utilizing a bacterial mutant with a defective form of one of the secreted components, EspA, that is unable to form a disulfide bond once secreted. The EspA mutant has decreased virulence during in vivo infection in mice and decreased mycobacterial cell wall integrity. Here, I tested the IFN β response, by both protein and mRNA techniques, to determine that the EspA disulfide-bond mutant was not defective in eliciting an innate immune response in macrophages.

2. *Critical role for invariant chain in CD1d-mediated selection and maturation of Va14-invariant NKT cells.*

Sillé FC, Martin C, Jayaraman P, **Rothchild A**, Besra GS, Behar SM, Boes M. . *Immunol Lett.* Sep 30;139(1-2):33-41.

3. *Requirement for Invariant chain in macrophages for Mycobacterium tuberculosis replication and CD1d antigen presentation.*

Sillé FC, Martin C, Jayaraman P, **Rothchild A**, Fortune S , Besra GS, Behar SM, Boes M. *Infect. Immun.* 2011 Aug;79(8):3053-63.

These two studies sought to determine the role of invariant chain (Ii) expression during *Mycobacterium tuberculosis* infection. Ii^{-/-} macrophages display early restriction of Mtb growth, yet delayed phagosomal maturation. Here, I assessed the rates of cell death and cytokine production in Ii^{-/-} macrophages compared to WT macrophages and found no effect of invariant chain expression on the cellular response of these macrophages to Mtb infection. In addition, these studies found that Ii^{-/-} macrophages do not upregulate CD1d expression and are unable to stimulate iNKT cells in co-culture to restrict bacterial growth. The invariant chain was shown to

play a role in iNKT cell development and maturation. iNKT cells from $li^{-/-}$ mice were defective in their cytokine responses and their ability to control Mtb growth in WT macrophages.

4. Recognition of microbial and mammalian phospholipid antigens by NKT cells with diverse TCRs.

Tatituri RV, Watts GF, Bhowruth V, Barton N, **Rothchild A**, Hsu FF, Almeida CF, Cox LR, Eggeling L, Cardell S, Rossjohn J, Godfrey DI, Behar SM, Besra GS, Brenner MB, Brigl M. *Proc Natl Acad Sci.* 2013 Jan 29;110(5):1827-32

This study characterized the lipid antigens recognized by diverse iNKT (dNKT) cells. I tested the recognition of various dNKT cell hybridomas to in vitro Mtb infection of RAW cells. I demonstrated CD1d-dependent recognition of Mtb-infected APCs by the dNKT cell hybridomas through measurement of IL-2 production. dNKT cells specifically recognized microbial phospholipid antigens found in Mtb and *Corynebacterium glutamicum* and this recognition required antigen presentation and processing by the APC but not separate TLR signaling. In addition, mammalian phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) also activated the dNKT cell hybridomas.

5. Interfering with mycobacterial metabolism synergizes with immune-mediated killing.

Zhang YJ, Reddy MC, Ioerger TR, **Rothchild AC**, Dartois V, Schuster BM, Trauner A, Valluru S, Huttenhower C, Sacchettini JC, Behar SM, Rubin EJ. *Cell* 2013 Dec 5;155(6):1296-1308.

In this study, a tryptophan production pathway was identified as a mycobacterial response to the $CD4^{+}$ T cell immune response through a transposon library screening technique (TraSH). Together, Jason Zhang and I tested the hypothesis that blocking tryptophan biosynthesis in Mtb, either using Mtb mutants or small molecule inhibitors, would inhibit bacterial growth and synergize with IFN γ -mediated protection during in vitro infection of murine macrophages.

6. *In search of a new paradigm for protective immunity to TB*

Nunes-Alves C, Booty MG, Carpenter SM, Jayaraman P, **Rothchild AC**, Behar SM. *Nat Rev Micro* 2014 Mar 3;12: 289-299.

In this review, we discussed recent discoveries in mechanisms of immune control during *Mycobacterium tuberculosis* infection, with special emphasis on CD4⁺ IFN γ -independent pathways that may be informative for future vaccine design. My contribution was focused on the role of pro-inflammatory cytokines and non-conventional T cells.